Response of a New Zealand tree fern (*Cyathea medullaris)* to Lead (Pb) treatments

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ABSTRACT

Lead (Pb) pollution is a serious environmental problem. Phytoremediation is emerging as a promising technology for removal of Pb and other heavy metals from soils and waterways. In this study, the phytoremediation potential of a popular landscape plant, in New Zealand, *Cyathea medullaris* (the black tree fern), was investigated. Pb uptake by the gametophytes and different parts (roots, shoots and leaves) of 3-month-old black tree fern plants in hydroponic experiments were studied using flame atomic absorption spectrometry. Morphological and ultrastructural changes in the Pb-treated materials were also investigated. Generally, the levels of Pb in the various black tree fern tissues increased with the external Pb concentration and exposure time. Transmission electron microscopy observation showed that the cell wall was the major subcellular site for Pb accumulation. Evidence obtained here suggests that the black tree fern gametophytes and the sporophyte can accumulate levels of Pb exceeding 1% of their dry weights. This resembles the capacity of a hyperaccumulating plant.

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$CaCl_2$	calcium chloride
dH_2O	distilled water
EDTA	ethylenediaminetetraacetic acid disodium salt
HNO_3	nitric acid
hrs	hours
min	minutes
ml	milliliters
MS	murashige and skoog nutrient medium
NO	nitric oxide
OsO_4	osmium tetraoxide
Pb	lead
$Pb(NO_3)_2$	lead nitrate
ppm	parts per million
ROS	reactive oxygen species
SNP	sodium nitroprusside
TEM	transmission electron microscope
v/v	volume per volume
w/v	weight per volume
μM	micromolar

CHAPTER 1.0

INTRODUCTION

1.1 Black Tree Fern

Cyathea medullaris (Forst.f) Swartz is a type of tree fern and also known as “Black Tree Fern”, “Sago Fern” and “Mamaku”. It belongs to the Cyatheaceae family and the Cyathea genus is native to New Zealand, Fiji and Polynesia (Braggins *et al.,* 2004). Among the NZ tree ferns, *Cyathea medullaris* is the tallest and fastest growing fern and it could reach a height up to 20 meters with frond length up to 6 meters (Braggins *et al.,* 2004). The preferred growing conditions for this fern are those found in a damp, moist partially shaded environment. It is a very adaptable and hardy plant even in less than ideal conditions. It is a beautiful garden plant and some parts are edible. The pith of the stem is very nutritious and is used raw or cooked (as sago substitute). The base of the frond stem and young croziers might be used as vegetables **(**Hedrick, 1972).

1.2 Heavy metals

Three main natural components soil, water and air are most important for any living matter in the world. Breaking the natural balance of one or all of these components could have disastrous consequences to the inhabitants in the environment. Presently every living thing is experiencing the polluted environment on a global scale (Nriagu, 1979). Due to the great amount of industrial activity throughout the last century, heavy metal contamination of the environment has become a serious dilemma. Many of these toxic metals enter the environment through fossil fuel combustion as well as mining and smelting processes (Bewley, 1980; Micera and Dessi, 1988). The natural process of metal transportation between the soil and water concentrates heavy metal contamination in the environment (Runnels and Shepherd, 1992). Once in the environment, metals are difficult to remediate. Among various pollutants, heavy metals have caused serious problems to the global biology. They are natural components of the Earth's crust and cannot be degraded or destroyed. Heavy metal contamination of soil, water and air has caused severe environmental problems because they tend to bioaccumulate in plants. This leads to complications in plants and indirectly affects human health through the food chain. It could

also directly affect human health (causing neurological diseases, genetic disorders and cancers) because heavy metals get into our bodies as trace elements through skin contact, drinking water and inhaling air (Nriagu, 1979).

Some heavy metals such as copper, iron, zinc and selenium are essential micronutrients for plant growth and development (Thornton, 1981). However, at higher concentrations, heavy metals in plants could act as stress factors imposing physiological constraints and bringing changes to various important growth processes such as photosynthesis, metabolism for normal plant growth, cell proliferation and differentiation of most plant cells (Raskin *et al.*, 1994). The mechanisms underpinning these effects are not very well understood yet.

1.3 Lead

Lead (Pb), is a heavy, bluish-grey metal, which has soft and malleable properties with a low melting point (Bell *et al.,* 1991). It does not occur naturally in pure form but it can be found in its divalent (2^+) oxidative state in ore deposits in the Earth's crust and globally distributed.

1.3.1 Sources of Pb

Pb is a heavy metal that has become the most important metal pollutant of the ecosystem (Salt *et al.,* 1998) due to the rapid increase of modern industries. Pb contamination has resulted from mining and smelting activities, effluents from storage batteries, fertilizers, pesticides, Pb-containing paints, gasoline, and explosives, as well as from the disposal of municipal sewage sludges enriched in Pb (Jackson and Watson, 1977; Levine *et al.,* 1998). Because many of the sources of Pb pollution are essential for modern human life, soil contamination with Pb is not likely to decrease in the near future. Figure 1 show various sources, which contribute to Pb pollution in the environment.

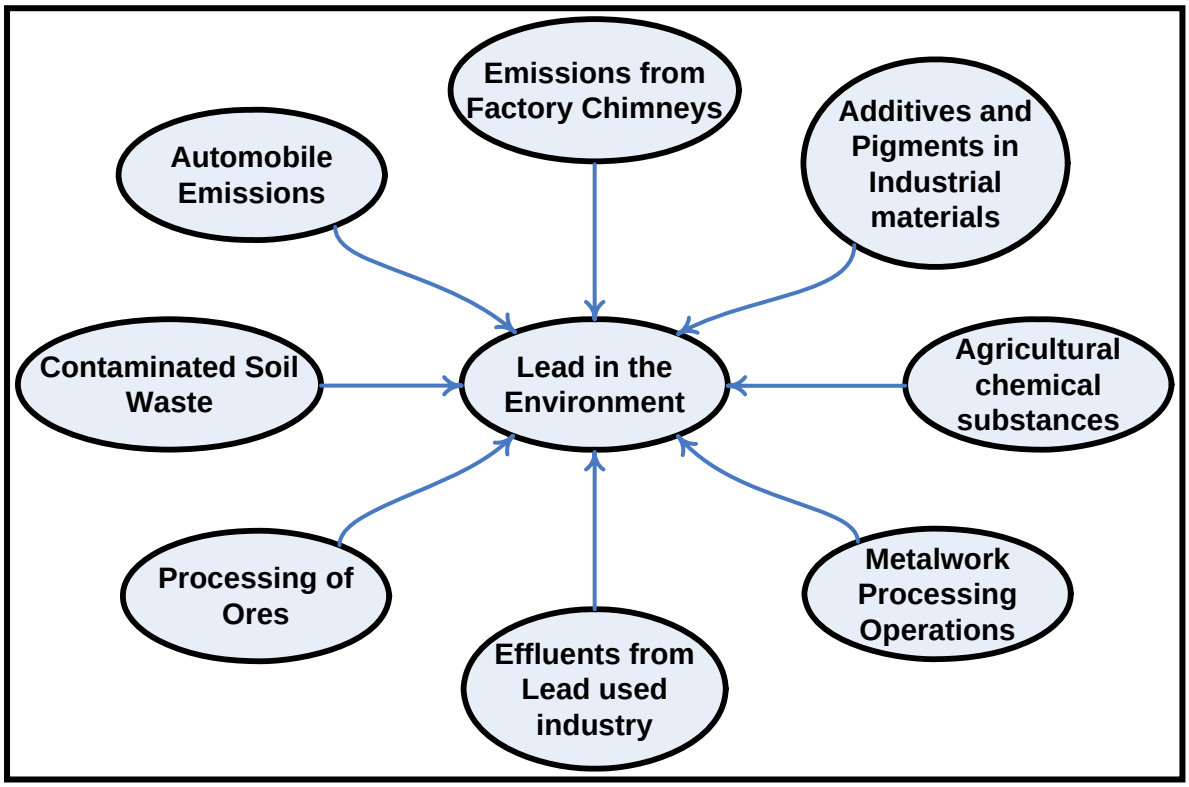

Figure 1: *Sources of Pb contamination into the environment*

1.3.2 Uses of Pb

There are many different uses of Pb, both as a metallic element and as its chemical compounds. For example, Pb is used in Pb acid batteries for electrical power storage, Pb sheets (or Pb clad steel) for building industry, Pb pipes for carriage of corrosive chemicals due to its corrosive resistant property, for joining Pb sheath electrical cables and in Pb sheathing for electric cables to reduce or stop corrosion under harsh environmental conditions. Also Pb is used in glass products (in stained glass windows, crystal glass) to give the lust, brilliance and density to the articles, in optical and window glasses and TV and monitor screens to filter out the radiation. There are too many uses of Pb to be all mentioned here and there would be many uses yet to be found.

1.4 Pb and Human health

Pb has so many industrial uses despite of its toxicity to the environment and to any living organisms. There are two ways that Pb can enter the human body, from mouth (ingestion and inhalation) and skin (broken skin, caused by an accident or purposely induced). Unborn and infant babies and children are the most vulnerable to be harmed by Pb

exposure because of their developing bodies (Carson *et al*., 1986)). Every system in the human body can be affected by Pb exposure and especially the developing brain and nervous system. High exposure to Pb could have very serious adverse effects on neurological, hematological, renal and reproductive systems. Once Pb enters the body, it travels through the blood to all soft tissues and organs, and then after several weeks it is stored in the teeth and bones by the body (as the body treats Pb as calcium). In adults, about 99% of Pb which enters the body will leave the system through waste (hair, nail, sweat, urine and feces) but in children, only about 32% will be eliminated. The harm done by Pb poisoning depends on the amount of Pb and the duration of the exposure (Adhikari *et al.*, 2001). Even in a small amount, Pb is very toxic and harmful to humans and animals (Figure 2).

Figure 2: *Assimilation of Pb into the Human body from the living environment and disposition of Pb from the body*

1.4.1 Pb and animals

Pb is a common cause of poisoning of domestic animals throughout the world (Liu, 2003). Non domestic animals such as cattle, especially young calves, are also extremely susceptible to Pb toxicity. However, Pb poisoning can occur in all domestic animals including horses, wild birds, poultry and dogs.

1.5 Plant and Pb

There is a widely held belief among researchers that Pb is very toxic to plants. Yet results of laboratory studies contradict field observations. There are actually no symptoms of Pb poisoning observed in plants growing under natural environment, even when such plants contain considerable amounts of Pb in their tissues (Koeppe, 1981).

However, in the 1960s some researchers examined the effects caused by Pb from car fumes (Cannon and Bowles, 1962) while others were interested in effects on food crops (Warren and Delavault, 1962). Schuck and Locke (1970) examined several crop species grown in close proximity to a busy highway and concluded that little translocation of Pb from root to shoot occurred. Miller and Koeppe (1971) determined Pb uptake in corn. This study confirmed that plants do have the ability to take up, translocation and accumulate significant amounts of Pb in leaves.

1.6 Pb uptake, transport and localization in plants

Pb is available to plants from soil, water and air. Studies of Pb uptake in plants have exhibited that roots have ability to take up significant quantities (Lane and Martin, 1977).

Pb accumulates in the surface layers of soil and therefore it is unreliable to measure a portion of soil where Pb is available to plants. Its availability depends on soil conditions and amount of Pb-binding organic material in the soil. Soil particle size and cation exchange capacity as well as plant factors such as root surface area, root exudates, mycorrhization and rate of transpiration affect the uptake of Pb (Davies, 1995).

At the root level, surface Pb binds to carboxyl groups of mucilage uronic acids. Mucilage binding restricts metal uptake into the root and establishes an important barrier protecting the root system. Some of the bound metals are released when mucilage is biodegraded (Morel *et al.*, 1986). Soil microorganisms may affect heavy metal availability by the process of biosorption, bioaccumulation and solubilization.

Pb retention in the roots is based on binding of Pb to ion exchangeable sites on the cell wall and extracellular deposition, mainly in the form of Pb carbonate deposited in the cell

wall. After being taken up by roots, the localization of Pb is higher in roots than in other parts of the plants. The addition of synthetic chelates, such as H-EDTA or EDTA, in combination with low pH, effectively prevents cell wall retention of Pb, making it available for translocation to shoots (Jarvis and Leung, 2001). Pb binds strongly to the carboxyl groups of the carbohydrates such as galacturonic acid and glucuronic acid in the cell wall, which restricts its transportation via apoplast (Rudakova *et al.,* 1988).

Pb transported from the soil to the root cells has to cross the root-cell plasma membrane. One possible transport pathway of Pb across the plasma membrane (PM) appears to be through PM cation channels, such as Ca-channels. A voltage gated Ca-channel in the root cell PM has been characterized using right-side-out PM vesicles isolated from roots of wheat and corn plants (Marshall *et al*., 1994; Huang *et al*., 1994). Huang and Cunningham (1996) found that Pb significantly inhibited voltage gated Ca-channels activity in the PM of wheat roots. The inhibition of the Ca-channel by Pb could arise from Pb blockage of the channel or due to competitive transport of Pb through the Ca-channel.

Pb moves predominantly into the root apoplast and then in a radial manner across the cortex and accumulates near the endodermis. The endodermis acts as a partial barrier to the movement of Pb between the root and shoot. This may in part account for the reports of higher accumulation of Pb in roots compared to shoots (Jones *et al*., 1973; Verma and Dubey, 2003). The limited transport of Pb from roots to other organs is due to the barrier of the root endodermis. It appears that the Casparian strip of the endodermis is the major limiting factor restricting Pb transport across endodermis into the central cylinder tissue (Seregin and Ivanov, 1997).

According to Lane and Martin (1977), the endodermis seems to act as a partial barrier because some of the Pb moves up through the vascular tissues and diffuses out into the adjacent tissues. This provides evidence that Pb moves into the symplast. The possibility of symplastic transport of Pb has been demonstrated in onion roots and garden cress hypocotyls (Wierzbicka, 1987). However, the movement of Pb in the root primarily via the apoplast is also supported by the report that a large proportion of Pb is readily extractable in water (Broyer *et al*., 1972).

Once metal ions have entered the roots, they can either be stored or exported to the shoot. Metal transport to the shoot primarily takes place in the xylem but there is some evidence

that redistribution may occur in the phloem (Stephan and Scholz, 1993). To enter the xylem, metal ions must first cross the Casparian strip, a layer of suberin-impregnated endodermal cells that divides the cortex and the vascular cylinder. To cross this strip of water-impermeable cell wall, metal ions must move symplastically within the endodermal cells, as apoplastic transport is blocked (Raven *et al.,* 1999). The possibility exists, therefore, that symplastic transport of metals within the endodermis is a rate-limiting step in metal translocation to the shoot. The involvement of organic acids in metal transport in certain metal accumulators has been demonstrated by analysis of xylem sap (Baker and Brooks, 1989).

Once trace metals accumulate within cells, they need to be detoxified and one of the ways this can occur is through compartmentalization. For example, Zn may be chelated to organic acids and accumulated within the vacuole (Mathys, 1977). Intact vacuoles isolated from tobacco exposed to Zn were shown to contain high levels of Zn (Krotz *et al.*, 1989). This has also been confirmed in roots and shoots of the Zn accumulator *Thlaspi caerulescens* (Vasquez *et al.*, 1994). Zn exposure can lead to an increase in the vacuolar volume fraction of meristematic cells within the root tip of *Festuca rubra* (Davies *et al.*, 1991).

Ultrastructural studies have shown that Pb taken up by plant roots tends to accumulate in the cell wall in *Allium cepa* (Antosiewicz and Wierzbick, 1999) and *Anthoxanthum odoratum* (Qureshi *et al.*, 1986). This ability of the cell wall to bind Pb is thought to play some role in Pb tolerance and this was confirmed by removal of the cell wall and exposure of the isolated protoplasts to Pb. Pb was also accumulated in vacuoles in *Allium* sp. (Antosiewicz and Wierzbick, 1999) and in tissue cultured cells of *Populus* sp.

Transition metals reach the apoplast of leaves in the xylem sap, from where they have to be scavenged by leaf cells. Transporters mediate uptake into the symplast, and distribution within the leaf occurs via the apoplast or the symplast. Trafficking of metals occurs inside every plant cell, maintaining the concentrations within the specific physiological ranges in each organelle and ensuring delivery of metals to metal-requiring proteins (Clemens *et al*., 2002).

1.7 Physiological, morphological and biochemical effects of Pb

Higher concentrations of both essential and non-essential heavy metals in the soil can lead to toxicity symptoms and the inhibition of growth of most plants. The toxicity symptoms seen in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular and molecular level. Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element resulting in deficiency effects (Van Assche and Clijsters, 1990).

The visible symptoms of Pb toxicity are rapid discoloration and inhibition of root growth, stunted growth of the plant and change in plant colour (chlorosis) (Burton *et al.*, 1984). When Pb enters the cells even in small amounts it causes a wide range of adverse effects on physiological processes. Pb toxicity leads to inhibition of enzyme activities, inhibition of photosynthesis, disturbed mineral nutrition, water imbalance, change in hormonal status and alteration in cell membrane structure and permeability. These disorders distress normal physiological activities of the plant. At high concentrations, Pb could lead to cell death (Ernst, 1998; Seregin and Ivanov, 2001). At the cellular level, Pb inhibits the activities of enzymes containing sulphydryl (-SH) groups necessary for their activity (van Assche and Clijsters, 1990). Pb toxicity inhibits germination of seeds and retards growth of seedlings. Pb decreases germination percentage, germination index, root and shoot length, tolerance index and dry mass of roots and shoots (Mishra and Choudhari, 1998). Pb toxicity lowers the protein content of tissues and causes significant alterations in lipid composition (Przymusinski *et al.*, 1991; Stefanov *et al.*, 1995). In *Phaseolus vulgaris* and *Zea mays* plants, substantial changes were observed in the level of glycolipids, especially monogalactosyl diacylglycerols, which are associated with membrane permeability in chloroplasts (Stefanov *et al.*, 1993).

1.7.1 Nutrient uptake

High concentrations of Pb in the soil cause imbalance of mineral nutrients in growing plants. Many of the observed actions of Pb appeared to be indirect because of mineral imbalance within the tissues. Significant changes in nutrient contents as well as in internal ratios of nutrients occur in plants under Pb toxicity in most cases, Pb blocks the entry of cations (K^+, Ca, Mg, Mn, Zn, Cu, and Fe^{3+}) and anions (NO_3^-) in the root system.

Pb physically blocks the access of many ions from absorption sites of the roots (Godbold and Kettner, 1991). Although Pb levels in root tips and the basal roots may appear to be similar, Pb changes the levels of mineral elements in the roots. For example, in root tips the levels of Ca, Fe, and Zn decrease after exposure to Pb.

1.7.2 Water status

A decline in transpiration rate and water content in tissues occurs in plants growing under Pb exposure. Various mechanisms have been suggested for the Pb-induced decline in transpiration rate and water content. Pb treatment causes growth retardation, which results in a reduced leaf area, the major transpiring organ (Iqbal and Moshtaq, 1987). Guard cells are generally smaller in plants treated with Pb. Pb lowers the level of compounds that are associated with maintaining cell turgor and cell wall plasticity and thus lowers the water potential within the cell. Metal ions including Pb increase the content of ABA and induce stomatal closure. Disordered respiration and oxidative phosphorylation observed under Pb toxicity may also cause confusion in the plant water regime.

1.7.3 Photosynthesis

The process of photosynthesis is adversely affected by Pb toxicity. Plants exposed to Pb ions show a decline in photosynthetic rate, which results from altered chloroplast ultrastructure, restrained synthesis of chlorophyll, plastoquinone, and carotenoids, obstructed electron transport, inhibited activities of Calvin cycle enzymes, as well as deficiency of CO_2 because of stomatal closure. *Ceratophyllum demersum* plants when grown in aquatic medium containing $Pb(NO_3)_2$ showed distinct changes in chloroplast fine structure (Rebechini and Hanzely, 1974). Leaf cells of such plants exhibited a reduction in

grana stacks. Pb treatment also changes the lipid composition of thylakoid membranes (Stefanov *et al*., 1995).

Pb inhibits chlorophyll synthesis by causing impaired uptake of essential elements such as Mg and Fe by plants (Burzynski, 1987). It damages the photosynthetic apparatus due to its affinity for protein N- and S- ligands (Ahmed and Tajmir-Riahi, 1993). An enhancement of chlorophyll degradation occurs in Pb-treated plants due to increased chlorophyllase activity (Drazkiewicz, 1994). Pb also causes strong dissociation of the oxygen evolving extrinsic polypeptide of PS II and displacement of Ca, Cl^-, and Mn from the oxygen-evolving complex (Rashid *et al*., 1991). Ahmed and Tajmir-Riahi (1993) found conformational changes in light-harvesting chlorophyll (LHC II) subunits, following binding with Pb *in vitro*. It is proposed that conformational changes induced by Pb treatment might lead to incomplete assembly followed by degradation (Ahmed and Tajmir-Riahi, 1993).

1.7.4 Respiration and ATP content

Pb has a significant effect on respiration and ATP content of photosynthetic organisms. *In vitro* application of Pb to mitochondrial preparations from plant cells revealed a decrease in respiration rate with increasing Pb concentrations (Reese and Roberts, 1985). Using isolated chloroplasts and mitochondria in different plant species, it has been shown that Pb affects the flow of electrons via the electron transport system (Miles *et al.*, 1972; Bazzaz, 1974). The inhibitory effect of Pb at higher concentrations seems to be due to uncoupling of oxidative phosphorylation (Miller *et al.*, 1973). At lower concentrations, however, a stimulation of respiration is observed in whole plants (Lee *et al*., 1976), detached leaves (Lemoreaux and Chaney, 1978), isolated protoplasts (Parys *et al*., 1998) and mitochondria (Koeppe and Miller, 1970). The exposure of detached leaves of plants (pea, barley) and plants (maize) to 5 mM $Pb(NO_3)_2$ for 24 hrs caused stimulation of the respiratory rate by 20-50 % (Romanowska *et al.*, 2002). Mitochondria isolated from Pb-treated pea leaves oxidized substrates (glycine, succinate, and malate) at higher rates than mitochondria from control leaves (Romanowska *et al*., 2002). Rapid fractionation of barley protoplasts incubated at low and high CO_2 concentrations indicated that the increased ATP/ADP ratio in Pb-treated leaves resulted mainly from the production of mitochondrial ATP. The activity of NAD^+-malate dehydrogenase in protoplasts of barley leaves treated with Pb was 3-fold higher than those from control leaves (Romanowska *et al*., 2002). The activities of

photorespiratory enzymes, NADH-hydroxypyruvate reductase and glycolate oxidase as well as of NAD-malic enzymes were however not affected by Pb treatment (Romanowska *et al*., 2002). The mechanism underlying the stimulation of respiration by some concentrations of Pb is not clear.

The inhibition of photosynthesis observed after Pb treatment leads to decreased utilization of ATP for CO_2 fixation. In leaf extracts of Pb-treated plants, higher ATP/ADP ratios have been observed compared to untreated plants (van Assche and Clijsters, 1990). Leaves of Pb-treated plants show increased respiration, which appears to be a result of oxidation of excess photosynthetic reducing equivalents, which are produced under conditions of limited CO_2 fixation (Poskuta *et al*., 1996).

At higher concentrations of Pb, inhibition of respiration is observed. Respiration of corn root tips decreased by 10-17 % after 1 h treatment with 20 mM $Pb(NO_3)_2$ and by 28-40 % after 3 hrs of treatment (Koeppe, 1977). Pb is regarded as one of the most potent metal ions for the inhibition of chloroplastic ATP synthetase/ATPase activity and for the destruction of the membranes (Tu Shu and Brouillette, 1987).

1.7.5 Enzymes activities

Pb treatment regulates the activity of a wide range of enzymes in different metabolic pathways. Pb at a concentration of 10^{-5} to 2 x 10^{-4} M brings about 50 % inhibition of many enzymes. In most, the inhibition exerted by Pb on enzyme activity results from the interaction of Pb with enzyme –SH groups (Levina, 1972). Pb interacts with free –SH groups that are present in the active site of the enzymes which are essential for enzyme activity as well as those that are essential for the maintenance of enzyme structure. In addition the reaction with –SH groups, blockage of –COOH groups with Pb ions also appears to play a major role in inhibition of enzyme activity under Pb treatment.

The key enzyme of chlorophyll biosynthesis, amino laevulinate dehydrogenase, is strongly inhibited by Pb ions (Prassad and Prassad, 1987). Pb also inhibits the activities of enzymes of the reductive pentose phosphate pathway (Hampp *et al*., 1973). In spinach leaves, the activity of ribulose-bis-phosphate carboxylase/oxygenase was inhibited even at a $Pb(NO_3)_2$ concentration of 5 µM (Vallee and Ulmer, 1972).

Plant species differing in Pb tolerance show varying behaviours of certain enzymes under Pb treatment. Igoshina and Kositsin (1990), while studying the effect of Pb on carboanhydrase activities in the tolerant and sensitive species of melic-grass (*Melica nutans*), observed that in tolerant melic-grass population, Pb activated carboanhydrase activity whereas in the sensitive plants the activity of this enzyme remained unaffected.

1.8 Phytoremediation

Phytoremediation is a term used for an assembly of technologies that use plants to reduce, remove, degrade, or immobilize environmental toxins (Salt *et al.,* 1998). To date, phytoremediation efforts have been focused on the use of plants to accelerate degradation of organic contaminants, usually in combination with root rhizosphere microorganisms, or remove hazardous heavy metals from soils or water.

Different plants absorb and bioaccumulate different levels of heavy metals. Phytoremediation is a bioremediation process that uses various types of plants to extract heavy metals and other pollutants from the contaminated environments, especially from soil and water. It has been understood that small cysteine-containing peptides have the capability of binding heavy metals called phytochelatins and metallothioneins (Grill *et al*., 1987). They are synthesized rapidly in some plant cells in response to high level of heavy metals. Some species of plants excrete specific organic substances from their roots, such as malate, histidine and citrate that have the ability to bind to heavy metals like copper, nickel and zinc and as a result, the metal-chelating compounds that are formed around roots could retard the absorption of other essential minerals into the plant cell (Cunningham *et al*., 1995; Salt *et al*., 1998).

Phytoremediation of polluted sites is extremely interesting because it is comparatively inexpensive. Seven aspects of phytoremediation have been described. These are phytoextraction, phytodegradation, rhizosphere degradation, rhizofiltration, phytostabilization, phytovolatization, and phytorestoration.

Phytoextraction

Phytoextraction involves the removal of toxins, especially heavy metals and metalloids, by the roots of the plants with subsequent transport to aerial plant organs (Salt *et al*., 1998;

Lombi *et al*., 2001). Pollutants accumulated in stems and leaves are harvested and removed from the site.

Phytovolatilization

Plants can also remove toxic substances, such as organics, from the soil through phytovolatization. In this process, the soluble contaminants are taken up with water by the roots, transported to the leaves, and volatized into the atmosphere through the stomata (Tollsten and Muller 1996; Newman *et al*., 1997).

Rhizosphere degradation

Like phytodegradation, rhizosphere degradation involves the enzymatic breakdown of organic pollutants, but through microbial enzymatic activity. These breakdown products are either volatilized or incorporated into the microorganisms and soil matrix of the rhizosphere. The types of plants growing in the contaminated area influence the amount, diversity, and activity of microbial populations (Jones *et al*., 2004; Kirk *et al*., 2005).

Rhizofiltration

Rhizofiltration removes contaminants from water and aqueous waste streams, such as agricultural run off, industrial wastes and nuclear material processing wastes (Salt *et al*., 1998). Absorption and adsorption by plant roots play a key role in this technique, and accordingly large root surface areas are usually required.

Phytostabilization

In phytostabilization, accumulation by plant roots or precipitation in the soil by root exudates immobilizes and reduces the availability of soil contaminants. Plants growing on polluted sites also stabilize the soil and can serve as a groundcover thereby reducing wind and water erosion and direct contact of the contaminants with animals (Kumar *et al*., 1995).

Phytorestoration

Phytorestoration involves the complete remediation of contaminated soils to fully functioning soils (Kumar *et al*., 1995). In particular, this subdivision of phytoremediation uses plants that are native to the particular area, in an attempt to return the land to its natural state.

1.8.1 Hyperaccumulation

The term, "hyperaccumulation" was first described by Jaffre *et al.*, (1976) when they found the New Caledonian plant *Sebertia acuminata* accumulated high levels of Ni in their above ground biomass. Hyperaccumulation is usually defined as the concentration of a metal ion to >0.1–1% of the dry weight of the plant (Baker, 1999). To date, approximately 400 plant species from at least 45 plant families have been reported to be hyperaccumulators. Most hyperaccumulators bioconcentrate Ni; about 30 absorb Co, Cu, and/or Zn; even fewer species accumulate Mn and Cd; and there are no known natural Pb-hyperaccumulators (Reeves and Baker, 1999).

However, several species, such as hemp dogbane (*Apocynum cannabinum*), common ragweed (*Ambrosia artemisiifolia*), nodding thistle (*Carduus nutans*), and Asiatic dayflower (*Commelina communis*), were shown to have superior Pb-accumulating properties (Berti and Cunningham, 1993). Practices have been developed to increase the potential of common nonaccumulator plants for Pb phytoextraction. Particularly, the uptake-inducing properties of synthetic chelates open the possibility of using high biomass-producing crops for Pb phytoextraction. Under chelate-induced conditions, maize (Huang and Cunningham, 1996) and Indian mustard (Blaylock *et al*., 1996) have been successfully used to remove Pb from solution culture and contaminated soil, respectively.

Metal hyperaccumulating plants are not only useful in phytoremediation, but also play an important role in biogeochemical exploring, and have implications on human health through the food chain (Boyd *et al.*, 1994).

The mechanisms of metal uptake by hyperaccumulating plants and the basis of their special ability are poorly understood. Metal hyperaccumulator plants are naturally talented for accumulating trace elements, mostly Ni, Zn, Cd, As or Se, in their above-ground tissues, without developing any toxicity signs (Baker *et al.*, 1994). There are many species of plants, which have the natural ability to absorb heavy metals and translocate them bioaccumulation. Hyperaccumulating plants are often common to naturally metal-rich soils. Among those plants, the fern *Pteris vittata* (brake fern) is found to be extremely efficient in extracting arsenic from soil and translocating it into its plant body. Probably, many more remain to be discovered (Kennelley, 2001).

Heavy metal contents in hyperaccumulators are at least 100 times those found in non-hyperaccumulating plants grown in soil under the same conditions (Rosselli *et al*., 2003). Several authors have suggested exploiting the unusual characteristic of these plants for the cleaning of metal-contaminated soils (Baker and Proctor, 1990).

In addition, several researchers investigated paths of increasing the biomass production of hyperaccumulating plants. Brewer *et al.,* (1999) reported the results of somatic hybridization of *T. caerulescens* with the high biomass crop *Brassica napus*. The hybrids obtained were taller and produced more biomass than *T. caerulescens*. They were able to accumulate Zn and Cd at levels that are toxic to *B. napus*, but below the levels commonly associated with hyperaccumulation (Brewer *et al.*, 1999). Another contemplated possibility is to use genetic engineering to confer the hyperaccumulation characteristics to high biomass plants (Pilon-Smits and Pilon, 2002). Although metal hyperaccumulator plants therefore appear to have ideal properties for phytoextraction, most of these plants produce little biomass and are thus primarily used as model organisms for research purposes (Walton and Anderson, 1992). However, the basic mechanisms for hyperaccumulation are still not fully understood.

1.9 Ferns for heavy metal extraction?

Ferns are ancient spore- forming vascular plants, which appear in the fossil record to have been around since 400 million years ago (Kendrick and Crane, 1997). Ferns, representing the highest evolutionary stage of vascular plants, occupy a middle branch of the vascular plant evolutionary tree (Raven *et al*., 1992). Like angiosperms, they have true leaves, which are a reflection of their comparatively advanced vascular systems (Banks, 1999). Ferns still maintain an independent haploid gametophyte generation evolved by their distinct antecedents, and hence, they are evolutionarily quite distinct (Raven *et al*., 1992). Most of the literature concerning metallophytes centered on angiosperms and other lower organisms, even though, ferns from metal-contaminated soils have been recognized. For instance, *Asplenium adulterium* is an indicator of nickel (Vogt and Quinitio 1994), while *Pellaea calomelanos* and *Chelianthes hirta* were found on copper and occasionally nickel soils (Wild, 1968). Ferns were also recorded on serpentine soils high in nickel and chromium (Kruckerberg, 1964). The degree to which fern species are restricted to serpentine varies widely. For instance, some ferns are apparently true endemics at the

species level, others are morphological and ecological variants of species possessing broader tolerance (Kruckeberg, 1964). Apart from terrestrial ferns, some aquatic ferns such as *Azolla siculoides* are able to take up large concentrations of heavy metals and accumulate in its shoots (Sela *et al*., 1989). Other ferns with metal accumulating capabilities include *Salvinia natans* for copper (Sen and Mondal, 1990), *Salvinia molesta, Azolla pinnath, Marsilea minuta* for cadmium, (Gupta and Devi, 1995), and *Salvinia minima* for chromium, (Nichols *et al*., 2000). Other than heavy metals, ferns have also been known to concentrate large quantities of trace elements in their tissues (Ozaki *et al*., 2000). The phenomenon of metal hyperaccumulation by plants has considerable importance in phytoremediation, more specifically phytoextraction (Rosselli *et al*., 2003).

The gametophyte of a fern, which is small and has a very simple structure without a vascular system, is independent of the sporophyte and autotrophic (Raghavan, 1989; Banks, 1999). This means that fern gametophytes have to adapt to the external environment to survive and produce sporophytes. In general, however, it seems that fern gametophytes are sensitive to stressful conditions because they are devoid of complicated protective devices, such as a thick cuticle on their epidermal cells (Raghavan, 1989). However it is known that a few ferns survive in extreme soil conditions that include high concentration of toxic metals (Honjo *et al*., 1980, 1984; Ma *et al.*, 2001; Francesconi *et al*., 2002), suggesting that their gametophytes are also tolerant to toxic concentration of heavy metals.

Honjo *et al.*, (1980, 1984) found that *Athyrium yokoscense* flourished on highly Pb-polluted soil where the concentrations of total Pb ranged from 3,000-57,000 μg g^{-1}. In addition, this fern has been shown to accumulate a large amount of Pb in the leaf blade (7.5-1,500 μg g^{-1}), petiole (64-1,000 μg g^{-1}) and rhizome and root (93-11,000 μg g^{-1}) all measured on the basis of fresh weight (Honjo *et al*., 1984). Thus, these results indicate that *A. yokoscense* might indeed be a Pb hyperaccumulator, which is defined as a plant containing over 1,000 μg g^{-1} of Pb in the dry matter (Baker and Brooks 1989). In this study, they found that the gametophytes of *A. yokoscense* are also tolerant to Pb toxicity, and could accumulate a high concentration of Pb, as in the case of the sporophytes.

1.10 Calcium and Pb toxicity

Calcium is an essential plant nutrient. As the divalent cation (Ca^{2+}), it is required for structural roles in the cell wall and membranes. It is required as a counter-cation for inorganic and organic anions in the vacuole, and as an intracellular messenger in the cytosol. Calcium enters plant cells through Ca^{2+}-permeable ion channels in their plasma membranes (White, 2000).

It was found that Pb caused numerous alterations in the apical cell of *Funaria hygyrometrica* protonemata. Pb deposits were found in the cell wall, endomembrane system, chloroplasts and nucle (Krzeslowska, 2004). It would be interesting to see if addition of calcium to Pb solution would also neutralise the toxic effects of the metal observed in the moss.

Calcium acts as the element that neutralizies the toxic effects of various stress factors (Abdelbasset *et al.,* 1995). Investigation of heavy metal tolerance in plants has given rise to evidence that calcium minimises uptake and toxicity of metals both from the soil (Cox and Rains, 1972; Simon, 1978) and from aqueous cultures (Baker, 1978). The regulation of calcium in the accumulation of particular ions from solution may be stimulatory (Maas *et al.,* 1969; Baker, 1978), but is more often inhibitory, particularly for heavy metals (Rashid, 1978). Baker (1978) examined the uptake of zinc from solution by zinc-tolerant and non-tolerant plants of *Selene maritime* while varying the calcium concentration. He found that increasing calcium led to greater zinc accumulation by the roots of the tolerant plants, but decreased transport of zinc to the shoots of both types of *Selene* plants.

1.11 Nitric oxide and Pb toxicity

Studies carried out during the last ten years have shown that the simple molecule nitric oxide (NO), acting in numerous metabolic areas. As it has been documented, NO controls the growth and development of the plant throughout its life, starting from germination (Beligni and Lamattina, 2000) and ending with flowering, ripening of its fruits and senescence of its organs (Leshem *et al*., 1998). Also under environmental stress conditions, both of abiotic and biotic origins, elevated generation of NO occurs in various organs of the plant (Del Rio *et al.*, 2004; Wandehenne *et al.*, 2004; Delledonne, 2005).

The effect of cytoprotective or cytotoxic action of NO on plant metabolism depends on the concentration of the molecule and is affected by the rate of synthesis, displacement and efficiency of removal of this reactive nitrogen species (Wojtaszek, 2000).

Nitric oxide donors, for example, sodium nitroprusside (SNP), are compounds, which produce NO when applied to the biological systems and are able to either mimic an endogenous NO-related response or substitute for an endogenous NO deficiency (Guo *et al*., 2004). The pathways leading to the formation of NO differ greatly among the various groups of plants, some of which require enzymatic catalysis, while others produce it non-enzymatically.

Transition metal NO complexes represent an important class of NO donors. The most commonly used is sodium nitroprusside (SNP), an NO^+ donor. Here NO acts as a powerful ligand, in which the nitrogen rather than oxygen binds the metal. The mechanism of NO release from SNP is not clear, although it has been known and used in the medical world for over 70 years. A SNP solution is extremely photosensitive and its degradation is promoted, by (for example) oxygen and temperature (Wang *et al*., 2002). According to the cited authors, NO release from the donor requires illumination or a single-electron reduction, which under physiological conditions may depend additionally on many reducing agents present in biological systems, such as, e.g. ascorbates, thiols, hemoproteins, as well as NADH and NADPH.

In relation to the above-mentioned issue numerous questions may be raised, namely whether the NO donor applied to a plant in different forms and at different doses, in the light and in the dark, can properly reflect functions fulfilled by endogenous NO in the tissue. It is a common opinion that current NO donors are normally thermodynamically unstable, especially in solutions and photosensitive (Leshem, 1996).

NO is involved in the signaling of growth, development, and adaptive responses to multiple stresses (Durner and Klessig, 1998) and in a number of cytotoxic and cytoprotective effects (Beligni and Lamattina, 2001) in plants. Not only do plants produce significant amounts of NO, but they also respond to atmospheric NO. NO action is achieved either directly, by reaction with effector molecules or indirectly, modifying the redox state of the cell. NO can readily form complexes with transition metal ions in aqueous solutions or those present in diverse nucleophylic compounds such as metalloproteins (Stamler *et al*., 1994).

1.12 Transmission electron microscopy

Transmission electron microscopy (TEM) is useful for analyzing metal and metal-compound specimens. It has been used to localize deposits and study effects of metal stress, especially Pb, in plants for many years. Early ultrastructural studies include those on *Pisum* chloroplasts (Sabnis *et al.,* 1969), *Avena* coleoptiles (Zegers *et al*., 1976), seeds of *Raphanus sativus* (Lane and Martin, 1977), and *Populus* tissue-culture cells (Ksiazek *and* Crane 1984).

1.13 Aims of study

An important goal in phytoremediation research is to seek "candidate" plants for the challenge of removing the pollutant in question. It would be appropriate to screen the plants in a geographical region for the suitability of phytoremediation potential pertinent to that region. In this study the popular landscape plant species, the tree ferns, were selected. In particular, the New Zealand black tree fern *(Cyathea medullaris)* was studied, as there is no prior similar study. Pb was used in the study because of its importance and persistence as an environmental pollutant.

The specific objectives of the present study are as follow:

1 To characterize the physiological and morphological changes of black tree fern and its gametophyte when exposed to different concentrations of Pb.

2 To localize Pb deposition within the black tree fern gametophyte and the tissues of the roots, shoots and leaves (frond) at the ultrastructural level.

3 To determine the effects of calcium on black tree fern gametophyte treated with toxic levels of $Pb(NO_3)_2$.

4 To determine the effects of nitric oxide on black tree fern gametophyte treated with toxic levels of $Pb(NO_3)_2$.

5 The two experiments above were designed to test the claims as reported that calcium and nitric oxide, when added to Pb treatment solutions, might mitigate the toxic effects of the Pb treatments in the published literature on other plant materials.

CHAPTER 2.0

MATERIALS AND METHODS

2.1 Plant materials

2.1.1 Spores

Spores of black tree fern *(Cyathea medullaris)* were obtained from New Zealand Tree Seeds (Rangiora, NZ). All the spores were stored at 4°C.

2.1.2 Plants

Two month-old-black tree fern plants were purchased from garden centre and maintained under glasshouse conditions at the University of Canterbury. They were grown in a soil less medium (Plate 1).

2.2 Germination of black tree fern spores under aseptic conditions

The spores were placed in a sterile vial (inside a laminar flow cabinet) with 10% (v/v) household bleach (containing 4.8 %, w/v, sodium (hypochlorite) and agitated for 10 min. They were then rinsed thoroughly three times with sterile distilled water. While being suspended in a small volume of sterile water solution, the spores were sown with a pipette onto 250 ml polycarbonate tissue culture containers (Labserv, Biolab, New Zealand) containing 30 ml of 1/10 strength of MS (Murashige and Skoog 1962) basal medium that was autoclaved before use (see 2.3.1). The containers were then placed in a growth room at 22°C with continuous lighting.

Plate 1: *3-month-old New Zealand black tree fern (Cyathea medullaris)*

2.3 Subculture of black tree fern gametophytes under aseptic conditions

After approximately 6 weeks, individual or pairs of gametophytes were transferred using a dissecting forceps and a scalpel, onto fresh medium under similar experimental conditions as for spore germination.

2.3.1 Preparation of media for spore germination and subculture

The MS medium used in this research was prepared as follows. Stock solutions of MS major salts, MS minor salts, MS organic and iron solutions were prepared (Appendix 1). The required constituents were dissolved in distilled water, combined, and the final volume adjusted. These stock solutions were stored at 4^{o}C while not in use. Media were prepared based on the appropriate experimental treatments. All media were adjusted to pH 5.7 before agar (Germantown N.Z. Co., N.Z.) at 8 g/L was added to the medium, heated in a microwave until the agar melted and dissolved. After this, the media (25 ml) were dispensed into autoclavable clear polycarbonate tissue culture containers (LabServ, New Zealand), and then autoclaved at 121^{o}C, 1.1 Kg cm^{-2}, for 20 min. All the autoclaved media were always left at room temperature for at least 24 hrs before use.

2.4 Detection of Pb (lead) uptake in black tree fern gametophytes

Black tree fern gametophytes grown under *in vitro* conditions were used for Pb uptake experiments. Six weeks after subculture, two pieces of the gametophytes that were of similar size were placed in a solution containing different concentrations of $Pb(NO_3)_2$. The concentrations of Pb solution tested were 0, 25, 50, 75, 100, 250, and 500 μM and five ml of one of these were placed in a McCartney glass vial (total capacity of 20 ml). The $Pb(NO_3)_2$ solutions were made up with nanopure water. No nutrients were supplied. Nanopure water was used as control. Observations were carried out daily, on gross morphological changes including gametophyte colour. The initial fresh weight was compared with that measured after exposure, and any physical differences observed were recorded. For each concentration of $Pb(NO_3)_2$, there were triplicate vials of gametophytes exposed to the Pb solutions for four weeks in a growth room at 22^{o}C with continuous lighting.

2.5 Detection of Pb uptake in 3-month-old black tree fern plants (Hydroponics experiment)

The three-month-old uniform plants were selected and removed from sand culture. The plants were washed carefully to remove the attached soil particles from their roots to prevent damage to intact root surface. The plants were placed in polycarbonate tissue culture containers with 150 ml of 0, 50,100, 250 or 500 μM of $Pb(NO_3)_2$ solutions prepared with nanopure water. The total volume of the solution was maintained the same by adding solution every two days and continuously aerated. There were three replicates in each treatment. No nutrients were supplied. Observations were carried out approximately every two days for signs of stress, color changes, growth inhibition and wilting of tissues.

2.6 Detection of calcium effects on Pb toxicity in gametophytes

Six-weeks-old *in vitro* grown uniform gametophytes were transferred to media containing different concentrations of $Pb(NO_3)_2$ with 10 μM $CaCl_2$ (Krzeslowska *et al*., 2004). The $Pb(NO_3)_2$ solutions were made up with nanopure water. No nutrients were supplied. The concentrations of Pb solution tested were 0, 50, 75 and 100 μM. These were placed in McCartney glass vials (each with a total capacity of 20 ml). The results were compared with control (5 ml of nanopure water only), 5 ml of $Pb(NO_3)_2$ samples, and along with 5 ml of 10 μM $CaCl_2$ samples. Observations were recorded daily, on gross morphological changes including gametophyte colour.

2.7 Detection of nitric oxide effects on Pb toxicity in gametophytes

Six-weeks-old *in vitro* grown uniform gametophytes were transferred to media containing different concentrations of $Pb(NO_3)_2$ with 10 μM sodium nitroprusside ($Na_2[Fe(CN)_5NO]\cdot 2H2O$, SNP) as a nitric oxide donor (Laspina *et al*., 2005). The $Pb(NO_3)_2$ solutions were made up with nanopure water. No nutrients were supplied. The concentrations of Pb solution tested were 0, 50, 75, 100, 250, and 500 μM. These were placed in McCartney glass vials (each with a total capacity of 20 ml). The results were compared with control (5 ml of nanopure water only), 5 ml of $Pb(NO_3)_2$ samples, and along with 5 ml of 10 μM SNP. Observations were recorded daily, on gross morphological changes including gametophyte colour.

2.8 Observation of Pb distribution in plant tissues

2.8.1 Fixation

Gametophyte, root, shoot, and leaf tissue sections of Pb-exposed and control plants were selected for microscopic analysis. Selected tissue sections were cut into smaller pieces by scalpel and placed in small glass vials in fixation buffer containing 3% glutaraldehyde (v/v) in 0.075 M sodium phosphate buffer, under partial vacuum, for 3 to 4 hrs at room temperature. The samples, in the same fixative, were then stored overnight at 4°C.

2.8.2 Post fixation

After the fixative was removed, all samples were washed twice, in 0.075 M phosphate buffer, for 10 min each time and then once more for 30 min. The specimens were then placed in full osmium tetroxide (OsO_4) in 0.075 M phosphate buffer, for 3 hrs, followed by a buffer change, overnight.

2.8.3 Dehydration

The samples were then dehydrated in small glass vials in an ethanol series of 20, 40, 60, and 80%, for 10 min at each step, followed by 100% ethanol, three times, each for 15 min.

2.8.4 Infiltration

In the first infiltration step the samples were placed in small glass vials in 1 part of Spurrs (Electron Microscopy Scientific Ltd., U.S.A.) resin: 2 parts of 100% acetone on a slowly rotating wheel overnight. The second infiltration step used 2 parts of Spurrs resin: 1 part of 100% acetone, on the wheel for at least 3 hrs.

2.8.5 Embedding

The samples were embedded in 100% Spurrs resin, in shallow plastic caps, placed in a glass Petri dish in a 60°C oven, overnight.

2.8.6 Ultramicrotomy

Right specimens were cut from the resin blocks and glued with epoxy resin to ‘stubs’ (OO capsules filled almost to the top with hardened epoxy resin). These were allowed to set in a 60ºC oven, overnight. The stubs were trimmed to produce flat surfaces exposing the desired part of the specimen. Once trimmed the stubs were mounted in an ultramicrotome (LKB 212B Ultratome) and approximately 90-100 nm ultra-thin sections were scraped and mounted on copper TEM grids.

2.8.7 Staining of ultra-thin sections

Most sections viewed in the TEM were unstained as adequate details were observed. However, some plant sections were stained for comparative purposes. These sections, still on the copper grids were placed in 1% uranyl acetate (v/v) in 50% ethanol for 10 min, followed by modified 3% (v/v) Reynold’s reagent (Hayat, 1975) containing three types of Pb compounds (nitrate, acetate, and citrate) in sodium citrate for 5 min.

2.8.8 Microscopy

Ultra-thin sections were viewed in a transmission electron microscope (Jeol-1200 EX) at an accelerating voltage of 80.0 kV. Micrographs were taken of cellular regions of interest on Kodak Estar electron microscopy sheet film (8.3 x 10.2 cm). Also distribution of the lead in the plant cell was investigated in an analytical TEM equipped with energy dispersive X –ray spectroscopy.

2.9 Analysis of Pb concentrations using a Varian AA-1475 series flame atomic absorption spectrometer (Jarvis and Leung, 2002)

All quantitative Pb analyses were carried out using a Varian AA-1475 series flame atomic absorption spectrometer. The machine was calibrated each time it was used with analytical grade 1000 ppm $Pb(NO_3)_2$ (BDH) diluted to 5, 10, 15, and 20 ppm in 1% HNO_3. In all trials, for all samples, three absorbance readings were taken at each level and the arithmetic means of these were estimated against calibration curves to provide Pb uptake levels in ppm.

2.9.1 Pb uptake in black tree fern gametophytes

The tree fern gametophytes were harvested after 7, 14 and 21 days of exposure to Pb treatment. After removal from solution, they were rinsed three times with dH_2O, desorbed for 30 min in 1.0 mM EDTA, dried overnight at 70°C oven. Fresh and dry weights of plant samples were recorded.

Silica crucibles were previously acid washed for 24 hrs in 10% HNO_3 (v/v), rinsed three time in dH_2O, and dried at room temperature. The dried plant materials were placed into crucible and ashed at 550°C for approximately 18 hrs. After cooling, 0.4 ml of 10% HNO_3 (v/v) with 1.6 ml nanopure water were added to the ash in each crucible, mixed thoroughly, and transferred quantitatively to labeled centrifuge tubes (previously acid washed for 24 hrs in 10% HNO_3 (v/v), rinsed three times in dH_2O, and dried at room temperature). These centrifuge tubes were stored in a refrigerator until analysis for lead concentrations using a Varian AA-1475 series flame atomic absorption spectrometer. All data were presented as mean values of three replicates.

2.9.2 Pb uptake in the 3-month-old black tree fern plants (from hydroponics experiments)

The plants were harvested after 7, 14 and 21 days of exposure to Pb treatment. After removal from solution, they were rinsed three times with dH_2O, desorbed for 30 min in 1.0 mM EDTA to remove lead accumulated on their surface. The plant were separated into roots, shoot and leaves (fronds) and dried overnight at 70°C oven (Plate 2). Fresh and dry

weights of plant samples were recorded and the samples were then ashed at 550°C for 18 hrs. After cooling, 1 ml of 10% HNO_3 (v/v) with 4 ml nanopure water were added to the ash in each crucible, mixed thoroughly, and transferred quantitatively to labeled centrifuge tubes (similar experimental condition as the detection of Pb uptake in fern gametophytes).

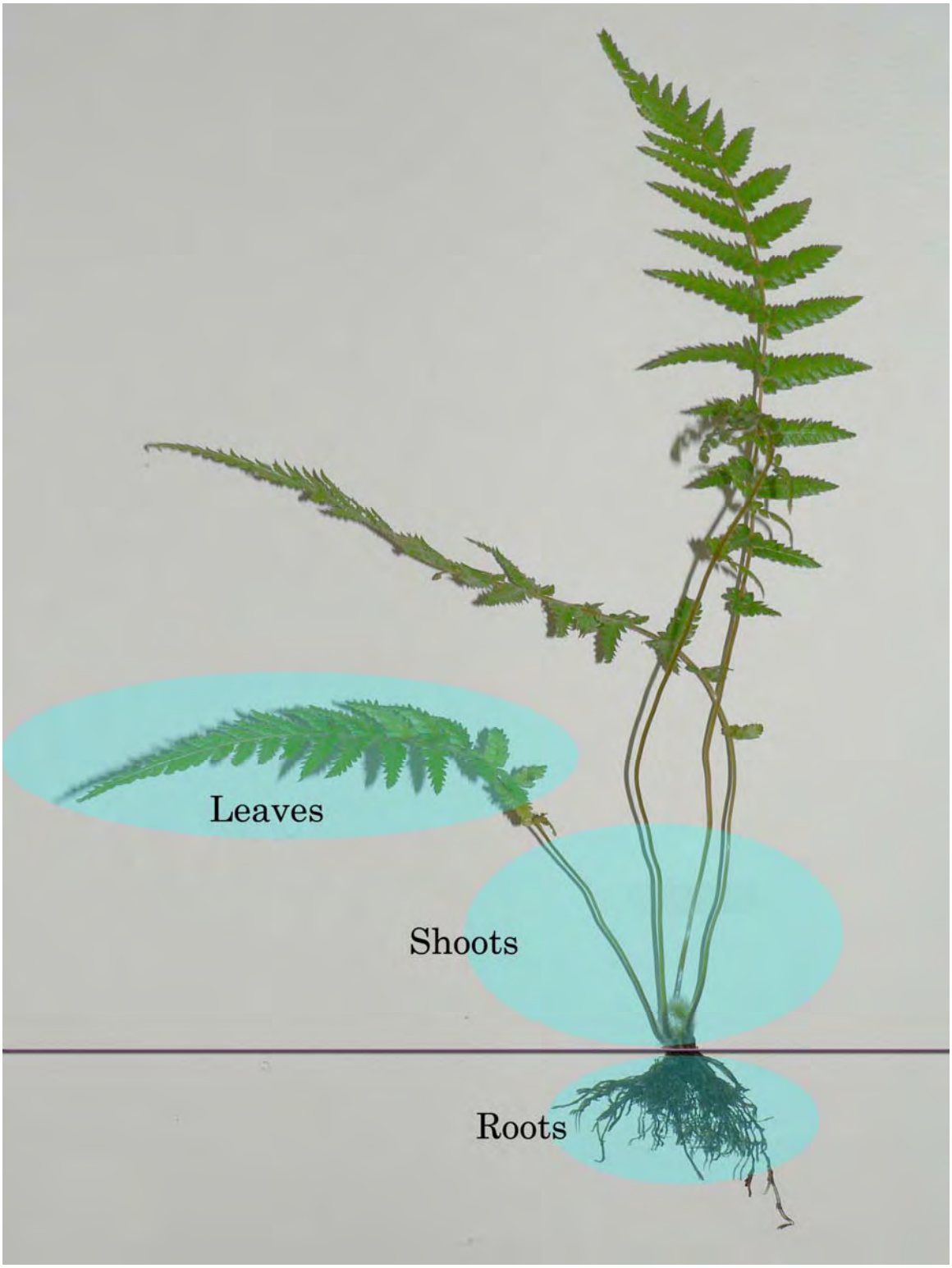

Plate 2: *Black tree fern's (Cyathea medullaris) roots, shoots and leaves used for analysis of Pb uptake*

2.10 Data analysis

All data were presented as mean values and standard deviations for three replicates. The statistical software package (Statistix, Version 8) was used. One way ANOVA analysis was used to determine whether the means were significantly different at $P<0.05$. Prior to ANOVA, all Pb uptake data were log 10 transformed to assure F max test homogeneity of variances. Two factor analyses of variance were carried out on data for: (a) root, (b) shoot and (c) leaf uptake of Pb. Multiple comparisons of means were performed using the Tukey test with standard error.

CHAPTER 3.0

RESULTS

3.1 Spore germination

Spore germination began within 3 - 4 weeks of sowing as a fine green “film” across the surface of the culture media. Each tiny green “speck” slowly developed into a flat, heart-shaped prothallus, usually 2 - 5 mm size across. Clump formation upon further development of the gametophytes was observed from the notch of the heart-shaped prothalli within 6 - 10 weeks.

3.2 Morphological changes of black tree fern gametophytes after exposure to Pb

Six-week-old gametophytes were grown in only nanopure water as a control. In addition, they were grown in different concentrations of Pb solutions. Before the imposition of experimental treatments, all gametophytes showed similar growth and developmental pattern. Following the initiation of Pb treatments, a number of visible differences in the gametophytes became apparent. Gametophytes supplied with 500 µM Pb developed dark staining around the prothalli after 3 days. The depth of the colour was greater, with increasing amount of Pb and the duration of exposure time. Those exposed to less than 500 µM Pb showed various morphological changes including stunted growth, browning or brown patches, shrinkage and stress (Table 1). The severity of the changes was greatest in gametophytes, which were exposed to 500 µM Pb, because complete death (necrosis) had occurred in most replicates within 15 - 18 days. Gametophytes exposed to Pb concentrations of less than 75 µM continued to develop normally after treatment had begun with the exception of some browning of the gametophytes. Those gametophytes supplied with 25 µM Pb, showed no adverse effects within 5 weeks. Samples in nanopure water (control samples) remained healthy (fresh-looking) and dark green throughout the 6 weeks of the experiment (Plate 3).

Weeks	1	2	3	4	5	6
Nanopure water	+++	+++	+++	+++	+++	+++
25 µM Pb	+++	+++	+++	+++	+++	++0
50 µM Pb	+++	+++	+++	++0	++0	++0
75 µM Pb	+++	+++	+++	++0	+00	000
100 µM Pb	+++	+++	++0	+00	000	000
250 µM Pb	+++	++0	+00	000	000	000
500 µM Pb	++0	+00	000	000	000	000

Table 1: *Response black tree fern of gametophytes (n=3) to different concentrations of $Pb(NO_3)_2$ over a period of 6 weeks*

KEY: [(+++ = Healthy, Green), (++0 = Stressed), (+00 = Dropping viability), (000 = dehydrated**,** dark brown)]

3.3 Fresh weight changes in gametophytes after exposure to Pb

The fresh weight of gametophytes can be used as a measure of the overall health of gametophytes growing in the presence of Pb. The influence of different Pb concentrations on the fresh weight of gametophytes is shown in Figure 3. There was little difference in the fresh weight of the gametophytes among the treatments in the first three days. In the control, there was a trend of increasing fresh weights up to day 9. The fresh weights of the gametophytes grown in both Pb solutions (250 and 500 µM) appeared to have decreased from day 6. The gametophytes in the control appeared to have noticeably higher fresh weights than those in the two Pb solutions from day 9.

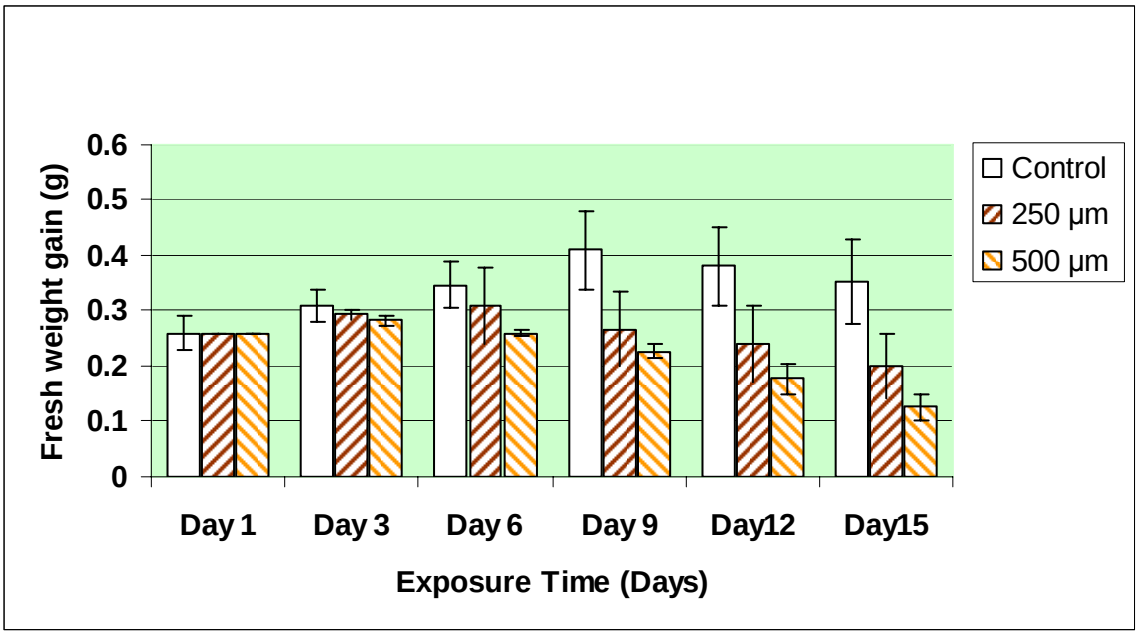

Figure 3: *Fresh weight changes in 6-week-old black tree fern gametophytes after exposure to different concentrations of $Pb(NO_3)_2$ for 2 weeks. Bars represent standard error (n=3)*

3.4 Morphological changes of three-month-old black tree fern plants after exposure to Pb

The results of growing black tree fern plants in various concentrations of Pb solutions are summarized in Table 2. In general, the results demonstrate that plant growth was likely to depend on the Pb concentration. For example, growth was inversely related to the parameter. Before the imposition of experimental treatments, all the plants were morphologically similar (Plate 1). Following the initiation of the Pb treatments, a number of morphological differences including stunted shoots and roots, were noticeable. In addition, there was a lack of root branching and root flaccidity. Plants supplied with Pb had developed a dark brown staining along the root length. The depth of the colour was greater with increasing supply of Pb and exposure time. Then with time, the plants started to dehydrate gradually. As a result, the plants seem to have shrunk with brown mottled, curled up leaves. Severity of the symptoms was highest in the plant, which had been exposed to 500 µM Pb. Complete death had occurred in most replicates of this treatment within 5 weeks. The plants exposed to 100 µM Pb or less, continued to develop normally for 4 weeks with the exception of some browning of the leaves.

Roots of all treated plants turned dark brown colour compared to the control. Control plants were able to develop new roots (lateral roots) and leaves (fiddleheads), and there as

no obvious chlorosis, withering or root discoloration. White root tips were noticed and they looked very healthy and vigorous throughout the whole experiment (Plate 4).

Weeks	**1**	**2**	**3**	**4**	**5**	**6**
Nanopure water	+++	+++	+++	+++	+++	+++
50 µM Pb	+++	+++	+++	+++	++0	000
100 µM Pb	+++	+++	+++	+++	+00	000
250 µM Pb	+++	+++	++0	++0	+00	000
500 µM Pb	+++	++0	++0	+00	000	000

Table 2: *Average response of black tree fern plants to different concentrations of* $Pb(NO_3)_2$ *over a period of 6 weeks (n=3)*

KEY: [(+++ = Healthy, Green), (++0 = Stressed), (+00 = Dropping viability), (000 = dehydrated)]

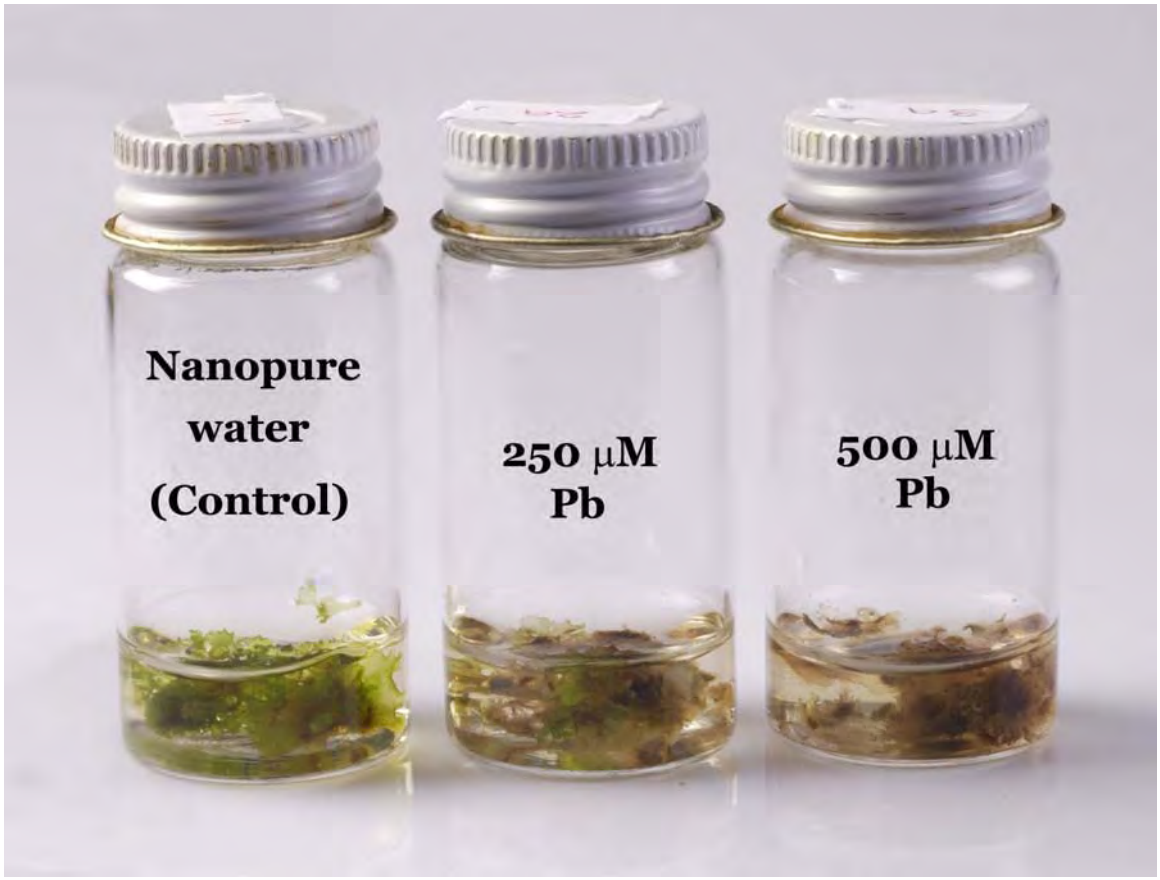

Plate 3: *Morphological changes of gametophytes in response to Pb treatments for 21 days*

Plate 4: *Morphological changes of 3-month-old black tree fern in response to Pb treatments for 21 days*

3.5 Pb uptake data: analysis of variance

1. All Pb uptake data passed the F (Max) test for homogeneity of variance.

2. The p-value from ANOVA tables were all less than 0.05 ($P < 0.05$).

3. Unplanned multiple comparisons of means were carried out using the Tukey-HSD test to determine which treatments were significantly different from each other. Treatments assigned with different letters are significantly different ($P < 0.05$).

3.6 Analysis of Pb concentrations in six-week-old black tree fern gametophytes

3.6.1 Overview

Pb contents of the Pb-treated plant tissues were determined quantitatively using flame atomic absorption spectrometry. Six-week-old black tree fern gametophytes grown under *in vitro* conditions were used for Pb uptake experiments. The presence of Pb in the ashes of the analyzed plant matter could be inferred from the appearance of the ash colour ranging from orange through to red. The colour intensity towards red indicates the high accumulation of Pb in the tissues. It was observed that the amount of Pb in the gametophytes increased with the increasing concentrations of Pb in the treatment solutions and exposure time.

3.6.2 Experiment 1a: Gametophytes treated with 0 (control), 25, 50, 75, and 100 µM of $Pb(NO_3)_2$ for 7 and 14 days

Among the different treatments, the gametophytes grown in 100 µM of Pb accumulated the highest level of Pb (2.80 mg of Pb per Kg of dry tissue) in 7 days (Figure 4). The Pb level in the control was very slight when compared to the other four Pb treatments.

After 14 Days, although there was an increasing trend of Pb uptake in relation to the increasing concentrations of Pb treatment solutions, there was no significant difference in the Pb level in those gametophytes treated with 50 µM or more of Pb (Figure 5).

By comparing the results in Figures 4 and 5, it can be clearly seen that longer exposure of the gametophytes resulted in greater Pb accumulation.

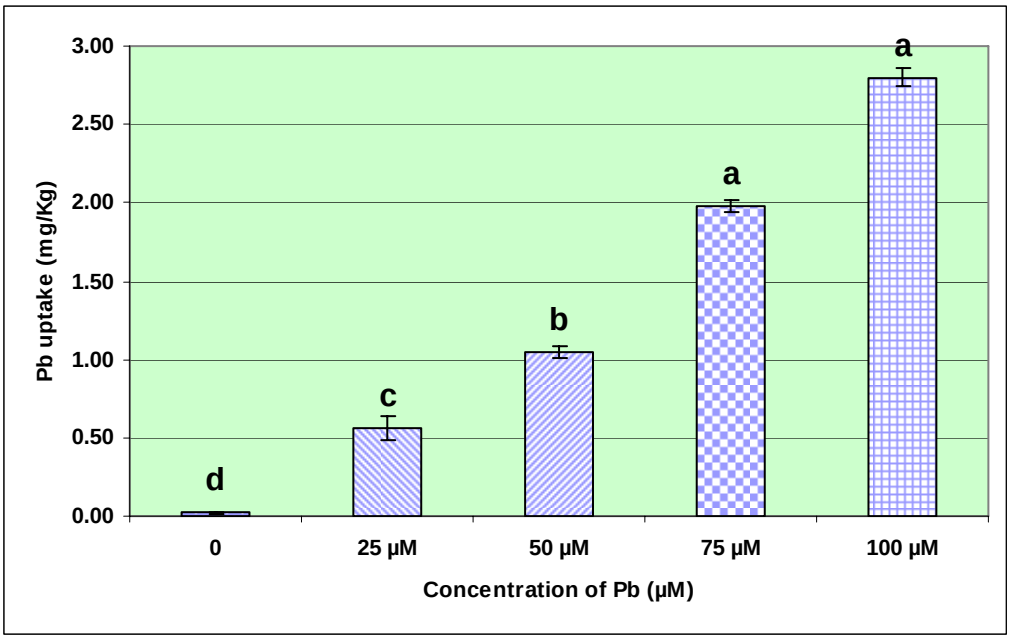

Figure 4: Pb concentration in six-week-old gametophytes of black tree fern *(Cyathea medullaris)* after exposure to different concentrations of Pb (0, 25, 50, 75 and 100 µM) for 7 days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

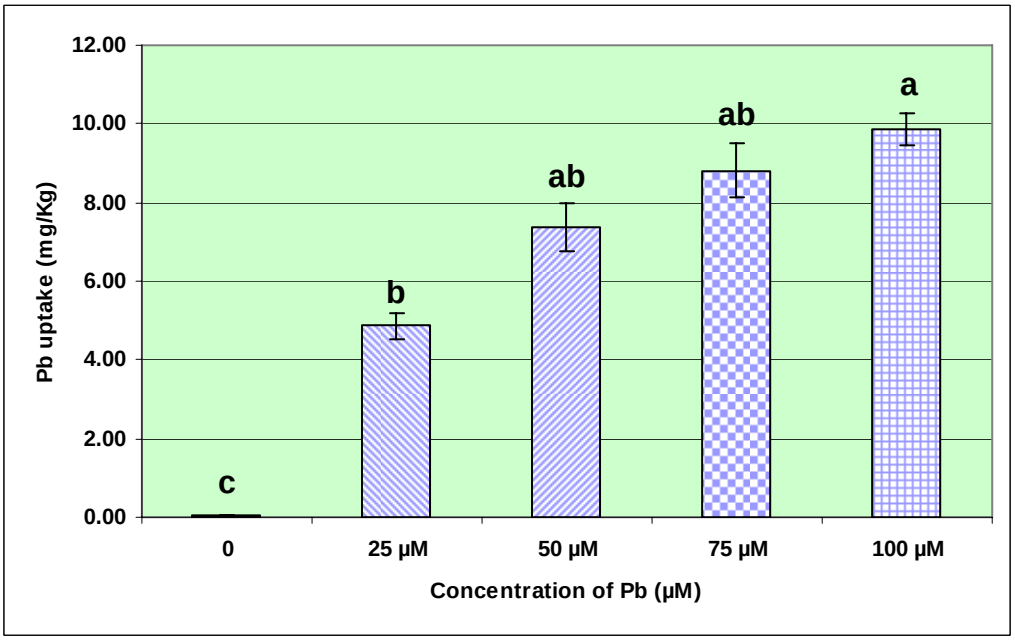

Figure 5: Pb concentration in six-week-old gametophytes of black tree fern *(Cyathea medullaris)* after exposure to different concentrations of Pb (0, 25, 50, 75 and 100 µM) for 14 days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

3.6.3 Experiment 1b: Gametophytes treated with 0 (control), 250 and 500 µM of $Pb(NO_3)_2$ for 7, 14 and 21 days

Gametophytes grown in 500 µM Pb, accumulated 9237.37 mg of Pb per Kg of dry tissue in 7 days, whereas those grown in 250 µM Pb, the Pb content was 5218.34 mg per Kg of dry tissue (Figure 6).

After 14 days, the gametophytes in both 250 and 500 µM Pb treatments continued to accumulate more Pb (Figure 7). Even though there was a trend showing that the 500 µM treatment resulted in greater accumulation of Pb in the gametophytes than the 250 µM treatment, there was no statistical difference between there two treatments.

After 21 days, the level of Pb accumulation in the 500 µM treatments (Figure 8) was similar to that after 14 days (Figure 7). At the same time, the gametophytes in the 250 µM treatment accumulated noticeably more Pb (18782.71 mg/Kg, Figure 8) than the level of Pb accumulated after 14 days (Figure 7).

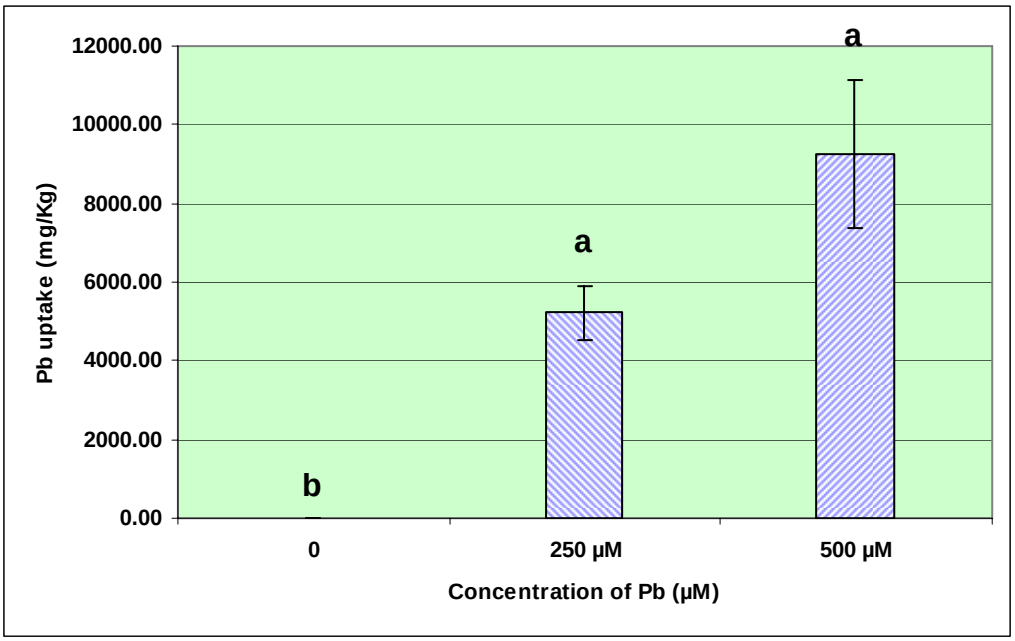

Figure 6: Pb concentration in six-week-old gametophytes of black tree fern *(Cyathea medullaris)* after exposure to different concentrations of Pb (0, 250 and 500 µM) for 7 days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

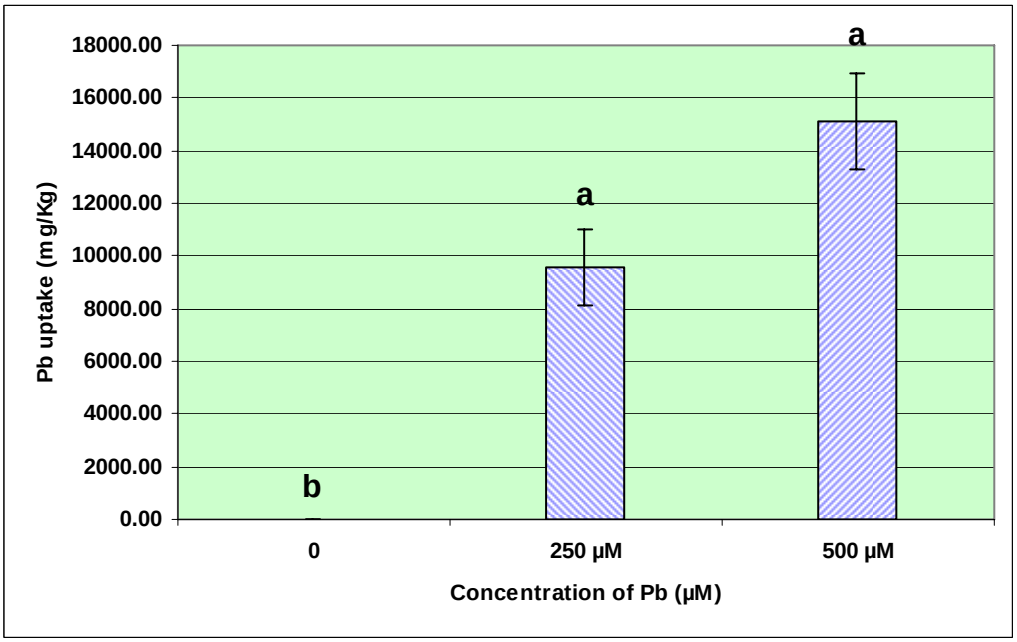

Figure 7: Pb concentration in six-week-old gametophytes of black tree fern *(Cyathea medullaris)* after exposure to different concentrations of Pb (0, 250 and 500 µM) for 14 days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

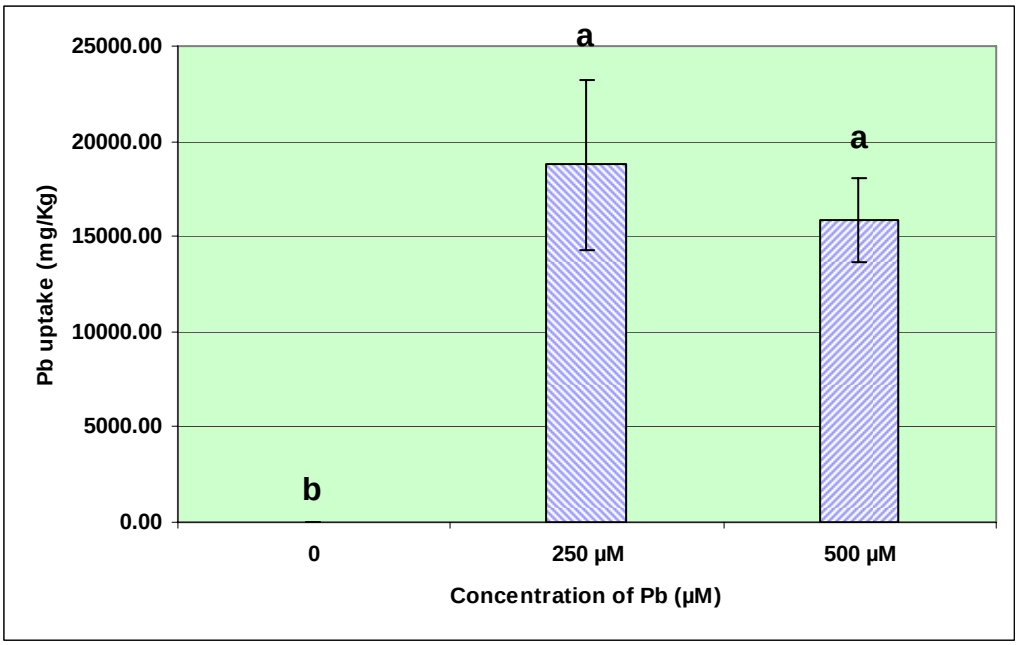

Figure 8: Pb concentration in six-week-old gametophytes of black tree fern *(Cyathea medullaris)* after exposure to different concentrations of Pb (0, 250, and 500 µM) for 21 days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

3.7 Analysis of Pb concentrations in 3-month-old black tree fern plants

3.7.1 Overview

There are some variations in Pb accumulation in various parts of the plant. It was observed that, regardless of the Pb concentration in growth media, root tissues always accumulated substantially more Pb compared to other parts of the plant with increasing exposure time (up to 21 days). It seems that the amount of Pb accumulated, in descending order, was roots, shoots and leaves, prior to 21 days. However, by 21 days, this behavior had changed and Pb accumulation in leaves was higher than that in shoots. This indicates Pb translocation from shoots to leaves.

3.7.2 Experiment 2a: Black tree fern plants treated with 0 (control), 50 and 100 µM of $Pb(NO_3)_2$ for 7 days

Generally, with more Pb in the growth solutions, there appeared to be more Pb accumulation in the roots, shoots and leaves of the black tree fern plants after 7 days of treatment (Figure 9). However, with the exception of Pb levels in the shoots, there was no statistical difference between the 50 and 100 µM Pb treatments.

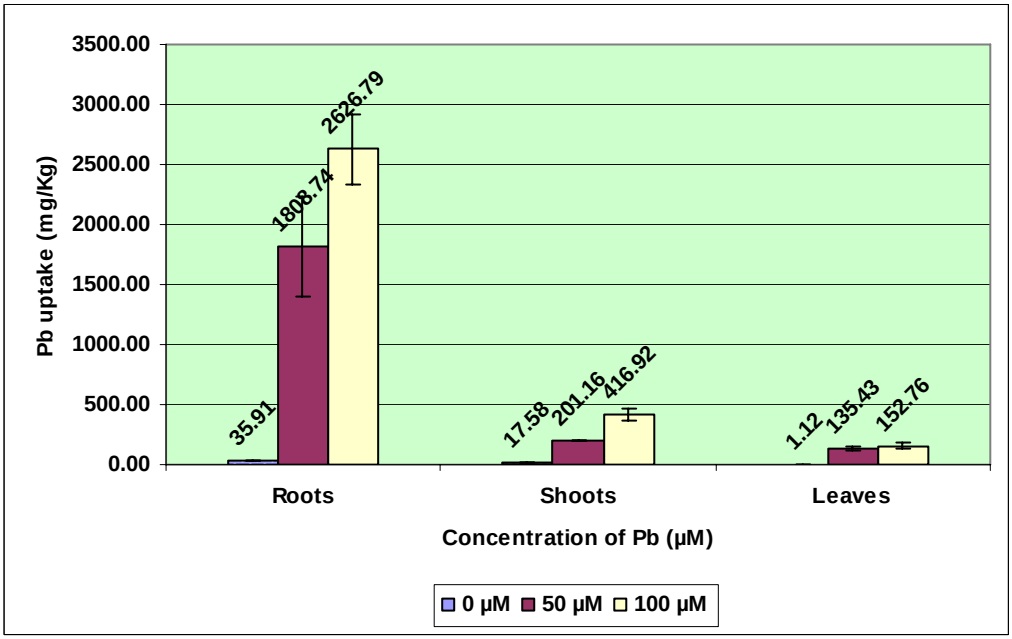

Figure 9: Pb concentration in roots, shoots and leaves of three-month-old of black tree fern *(Cyathea medullaris)* plants after exposure to different concentrations of Pb (50 and 100 µM) for 7 days. Data are means of three replications, and bars depict ± SE. Treatments with different letters are significantly different ($P < 0.05$).

3.7.3 Experiment 2b: Black tree fern plants treated with 0 (control), 50 and 100 µM $Pb(NO_3)_2$ for 14 days

After 14 days of treatment, exposure to 100 µM Pb resulted in significantly more Pb accumulation then exposed to 50 µM Pb in the roots and shoots, but not leaves of black tree fern plants (Figure 10). In the 50 µM treatment, longer treatment time did not seem to result in more Pb accumulation in the roots (compared with Figures 9 and 10).

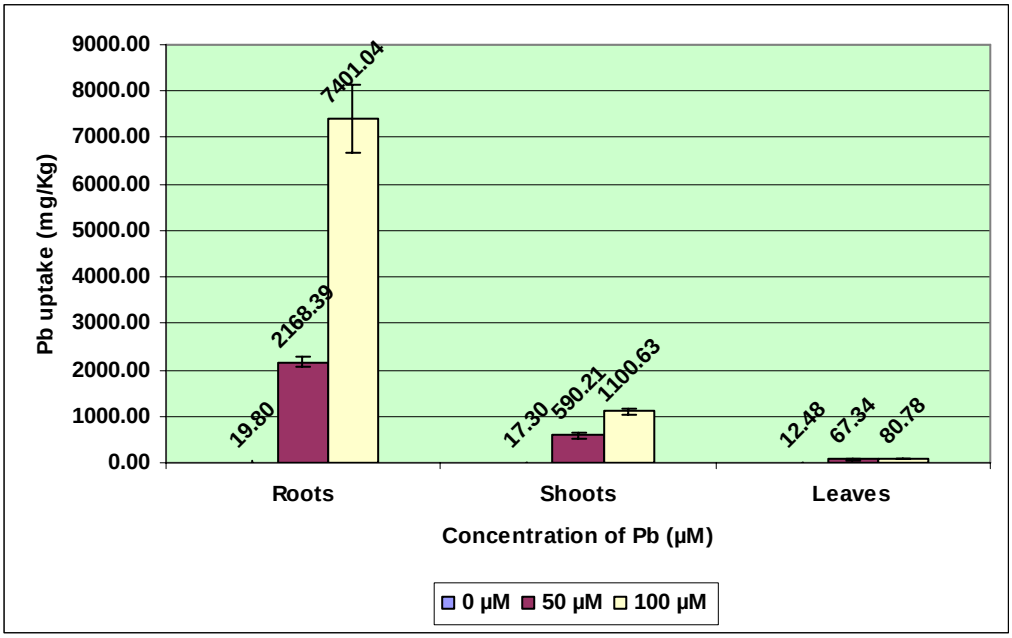

Figure 10: Pb concentration in roots, shoots and leaves of three-month-old of black tree fern *(Cyathea medullaris)* plants after exposure to different concentrations of Pb (50 and 100 µM) for 14 days. Data are means of three replications, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

3.7.4 Experiment 3a: Black tree fern plants treated with 0 (control), 250 and 500 µM of $Pb(NO_3)_2$ for 7 days

When the Pb concentrations in the growth media were 250 and 500 µM, the Pb levels of root tissues were 17337.17 and 19258.68 mg/Kg root dry weight, respectively, after 7 days of treatments (Figure 11). It can be clearly seen that these levels were dramatically higher than those in the treatments with 100 µM or less Pb for (Figure 9) the same period of time (7 days). For example, the 500 µM Pb treatments resulted in about 9 times more Pb accumulation than the 100 µM Pb treatments (compared Figure 9 and 11). There was no significant difference between the 250 and 500 µM Pb treatments did not result in significantly different levels of Pb in the roots, or shoots or leaves (Figure11).

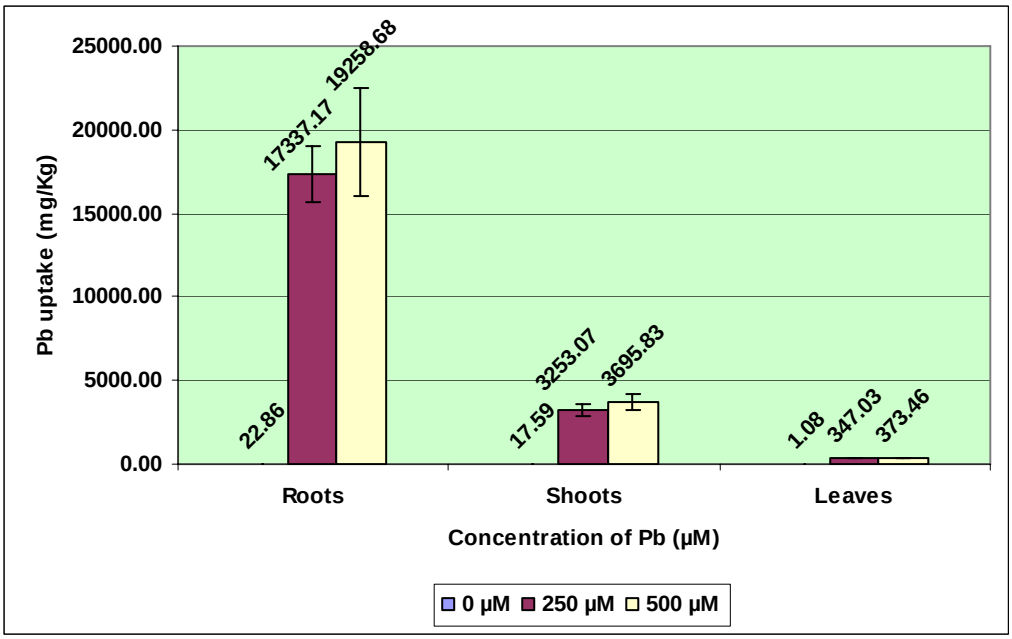

Figure 11: Pb concentration in roots, shoots and leaves of three-month-old of black tree fern *(Cyathea medullaris)* plants after exposure to different concentrations of Pb (0, 250 and 500 µM) for 7 days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

3.7.5 Experiment 3b: Black tree fern plants treated with 0 (control), 250 and 500 µM of $Pb(NO_3)_2$ for 14 days

After 14 days there, were significant differences between 250 and 500 µM of Pb treatments, affecting contents of, the roots and leaves, but not the shoots (figure 12). In all three plant tissues analyzed, more Pb accumulation was found than 7 days earlier.

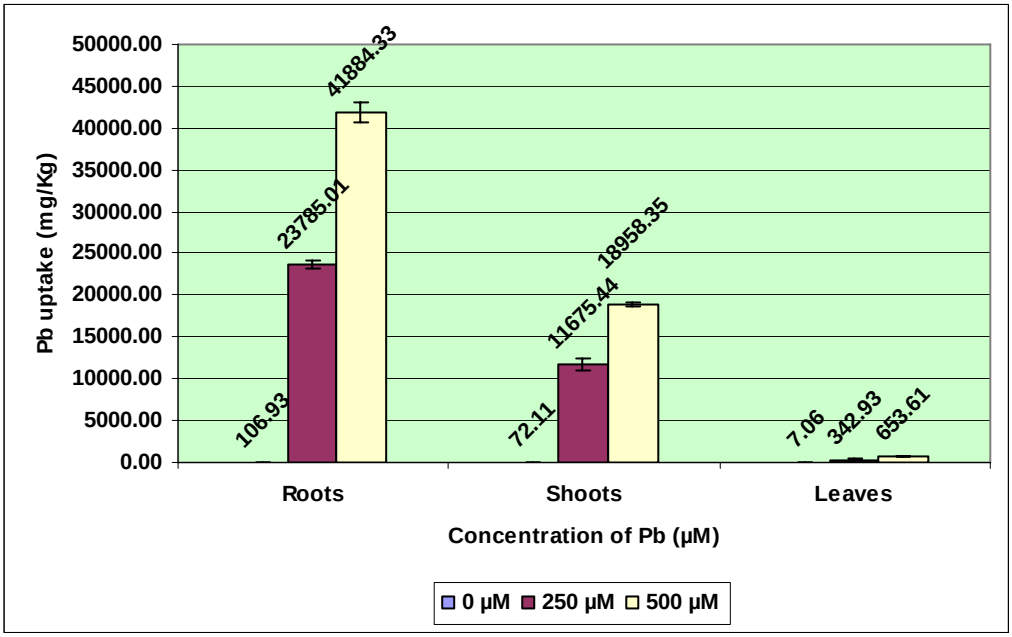

Figure 12: Pb concentration in roots, shoots and leaves of three-month-old of black tree fern *(Cyathea medullaris)* plants after exposure to different concentrations of Pb (0, 250 and 500 µM) for 14 days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

3.7.6 Experiment 3c: Black tree fern plants treated with 0 (control), 250 and 500 µM of $Pb(NO_3)_2$ for 21 days

It seems that there was a small or no further increase in the Pb contents of the roots of black tree fern plants after 14 days of treatment with 250 or 500 µM Pb (compare Figures 12 and 13). For Pb accumulation in the shoots, there was a significant difference between 250 and 500 µM Pb treatments (Figure 13). It appears that the Pb level in the shoots at day 21 was lower than that at day 14 (compare Figures 12 and 13). Conversely, the Pb level in the leaves at day 21 was several times higher than that of day 14 (compare Figures 12 and 13).

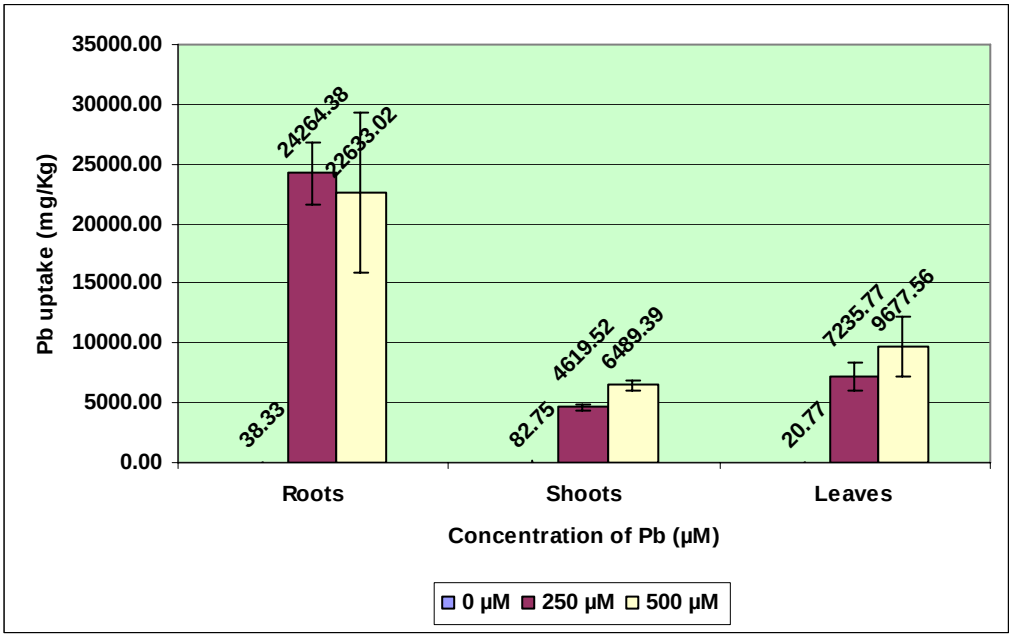

Figure 13: Pb concentration in roots, shoots and leaves of three-month-old of black tree fern *(Cyathea medullaris)* plants after exposure to different concentrations of Pb (0, 250 and 500 µM) for 21days. Data are means of three replicates, and bars depict ± SE. Treatments with different letters are significantly different (P < 0.05).

3.8 Effect of calcium on Pb toxicity in black tree fern gametophytes

Six- week-old black tree fern gametophytes grown from spores under *in vitro* conditions were transferred to media containing different concentrations of $Pb(NO_3)_2$ with or without 10 µM $CaCl_2$. Gametophytes treated with 25 µM Pb started to show stress after 5 weeks of treatments (Table 3). This was apparently prevented in the presence 10 µM $CaCl_2$. This concentration of $CaCl_2$ did not have large effect on the deleterious action of 50 µM or higher concentrations of $Pb(NO_3)_2$ on black tree fern gametophytes. In fact, the results suggest the plants became unhealthier.

WEEK	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
NANOPURE WATER	+++	+++	+++	+++	+++	+++	+++	+++
25 µM Pb	+++	+++	+++	+++	+++	++0	+00	000
50 µM Pb	+++	+++	+++	++0	++0	000	000	000
75 µM Pb	+++	+++	+++	++0	000	000	000	000
100 µM Pb	+++	+++	++0	000	000	000	000	000
10 µM $CaCl_2$	+++	+++	+++	+++	+++	+++	+++	+++
25 µM Pb + 10 µM Ca^{2+}	+++	+++	+++	+++	+++	+++	+++	+++
50 µM Pb + 10 µM Ca^{2+}	+++	+++	+++	+++	++0	+00	000	000
75 µM Pb + 10 µM Ca^{2+}	+++	+++	++0	000	000	000	000	000
100 µM Pb + 10 µM Ca^{2+}	+++	000	000	000	000	000	000	000

Table 3: Response of the gametophytes (n=3) to different concentrations of $Pb(NO_3)_2$ with or without $CaCl_2$.

KEY: [(+++ = Healthy, Green), (++0 = Stressed), (+00 = Dropping viability), (000 = dehydrated, dark brown)]

3.9 Effect of Nitric oxide on Pb toxicity in black tree fern gametophytes

Six-week-old black tree fern gametophytes grown from spores under *in vitro* conditions were transferred to media containing different concentrations of $Pb(NO_3)_2$ with or without 10 µM sodium nitroprusside (SNP) a nitric oxide donor. Results are shown Table 4. When SNP was added to 25-75 µM $Pb(NO_3)_2$, SNP exhibited protective effect on the black tree gametophytes. Addition of SNP also improved the condition of the gametophytes treated with 100 µM Pb. However, SNP could not counteract the harmful effect of 250 µM of $Pb(NO_3)_2$.

WEEK	**1**	**2**	**3**	**4**	**5**	**6**	**7**	**8**
NANOPURE WATER	+++	+++	+++	+++	+++	+++	+++	+++
25 µM Pb	+++	+++	+++	+++	+++	++0	+00	000
50 µM Pb	+++	+++	+++	++0	++0	000	000	000
75 µM Pb	+++	+++	+++	++0	000	000	000	000
100 µM Pb	+++	+++	++0	000	000	000	000	000
250 µM Pb	+++	++0	000	000	000	000	000	000
10 µM SNP	+++	+++	+++	+++	+++	+++	+++	+++
25 µM Pb + 10 µM SNP	+++	+++	+++	+++	+++	+++	+++	+++
50 µM Pb + 10 µM SNP	+++	+++	+++	+++	+++	+++	+++	+++
75 µM Pb + 10 µM SNP	+++	+++	+++	+++	+++	+++	+++	+++
100 µM Pb + 10 µM SNP	+++	+++	+++	+++	+++	++0	+00	000
250 µM Pb + 10 µM SNP	+++	+00	000	000	000	000	000	000

Table 4: Response of the gametophytes (n=3) different concentrations to $Pb(NO_3)_2$ with or without 10 µM SNP in 2 month.

KEY: [(+++ = Healthy, Green), (++0 = Stressed, part of more green), (+00 = Dropping viability, many parts became brown), (000 = dehydrated, dark brown)]

3.10 Use of transmission electron microscopy for ultrastructural observations of Pb-treated plant cells:-

Ultrastructure of gametophytes of black tree fern (*Cyathea medullaris*): unstained section from control (incubation in water only for 3 days, Plate 5).

The cells shown in (Plate 5) appeared highly vacuolated and fully mature. Organelles were clearly distinguishable. The mitochondria and chloroplasts with starch grains were well defined. No Pb deposits were observed in these cells (Plate 5).

Ultrastructure of gametophytes of black tree fern (*Cyathea medullaris*): unstained section from (incubation in 250 µM Pb for 3 days, Plate 6).

The epidermis cells contained large mature vacuoles. The flattened chloroplasts with starch grains were within the mature vacuolated cells in a centrifugal position. A small amount of fine Pb particles could be seen in the cell wall and cytoplasm (Plate 6).

Ultrastructure of gametophytes of black tree fern (*Cyathea medullaris*): unstained section from (incubation in 500 µM Pb for 3 days, Plate 7a and Plate 7b).

Several cells contained large irregular mature vacuoles. Numerous oblong chloroplasts were displayed in the mature vacuolated cells in a centrifugal position. In the cytoplasm, chloroplasts with starch grains and, mitochondria were clearly defined. A small amount of Pb particles could be seen in the intercellular region and adjacent the wall (Plate 7a).

The plasma membranes seemed to be detached from the cell wall and numerous Pb deposits could be observed in the space between the cytoplasm and plasma membrane. In remnants of cytoplasm at higher magnification, degraded chloroplasts with broken membranes and starch grains were observed (Plate 7b).

Ultrastructure of gametophytes of black tree fern (*Cyathea medullaris*): unstained section from control (incubation in water only for 21 days, Plate 8).

The cells appeared highly vacuolated and fully mature. The cell wall was normal in appearance. The oblong chloroplasts with starch grains and mitochondria were well defined. No deposits of Pb were found in these gametophytes (Plate 8).

Ultrastructure of gametophytes of black tree fern (*Cyathea medullaris*): unstained section from (incubation in 250 µM Pb for 21 days, Plate 9).

At 5,000X magnification, the cells appeared highly vacuolated and fully mature. Relatively large Pb deposits were observed in the surface between cytoplasm and cell walls (Plate 9).

Ultrastructure of gametophytes of black tree fern (*Cyathea medullaris*): unstained section from (incubation in 500 µM Pb for 21 days, Plate 10a and Plate10b).

The largest and most numerous Pb deposits were accumulated in surface between cytoplasm and plasma membrane. The thickness of cell walls increased noticeably (Plate 10a).

Cells contain large vacuoles and many fine Pb particles were precipitated in intercellular region (Plate 10b).

Ultrastructure of gametophytes of black tree fern (*Cyathea medullaris*): stained section from (incubation in 500 µM Pb for 21 days, Plate 11a and Plate 11b).

At 15,000X or higher magnification, the cell contained large vacuoles. The thickness of cell walls increased noticeably and big Pb deposits were found in between the cytoplasm and plasma membrane (Plate 11a). A relatively large accumulation of Pb particles was found in the middle lamella and intercellular region (Plate 11b). By comparing with unstained and stained ultra thin section of tree fern (*Cyathea medullaris*) gametophytes treated with 500 µM of $Pb(NO_3)_2$ for 21 days there is no any differences were observed.

Non-Pb treated tree fern (*Cyathea medullaris*): Unstained section of root tissue (Plate 12).

At 8,000X magnification, in the cytoplasm of mature vacuolated cells, the cytoplasm contained numerous organelles were present. The cytoplasm was apparently rich in vacuoles, vesicles, starch grain, plastid and small plastoglobuli and endoplasmic reticulum. No Pb deposits were found in these root tissue controls (Plate 12).

Root tissue from the treatment with 250 µM Pb: Unstained section (Plate 13a and Plate 13b).

At 12,000X or higher magnification, in the epidermal cells of the Pb-treated root tissue the vacuoles were large and of irregular shape. Many vesicles of different sizes appeared between the peripheral layers of the cytoplasm (Plate 13a).

Damaged membranes of organelles and severe plasmolysis with separations of the plasma membrane from the cell wall were observed in most root cells. Small electron-dense Pb deposits were observed in the damaged organelles and inside vacuoles (Plate 13b).

Root tissue from the treatment with 500 µM Pb: Unstained section (Plate 14a, Plate 14b and Plate 14c).

The root cells exhibited various ultra-structural changes when compared to control root cells. The cytoplasm was severely degraded so that the organelles could not be properly identified. Large Pb deposits were observed in remnants of the cytoplasm (Plate 14a).

Drops or clumps of dense Pb deposits were seen in to the cell wall, scattered in the middle lamella and accumulated in the intercellular space (Plate 14b).

The cell wall was often lined with large electron dense Pb deposits. They formed a layer of varying width between the cell wall and remnants of cytoplasm (Plate 14c).

Non-Pb treated tree fern (*Cyathea medullaris*) shoot tissue: Unstained section (Plate 15).

These cells appeared highly vacuolated and fully mature. Mitochondria, chloroplasts with grana, as well as stroma and thylakoids, starch grains were observed in the cytoplasm. No Pb deposits were observed in theses control tissues.

Shoot tissue from the treatment with 250 µM Pb: Unstained section (Plate 16a and Plate 16b).

A thin layer of cytoplasm contained numerous oblong chloroplasts with thylakoids, starch grains, small plastoglobuli and multiple mitochondria (16a). Occasional small or larger Pb particles were seen outside the cell wall. Pb particles were not observed in the cytoplasm at a higher magnification (Plate 16b).

Shoot tissue from the treatment with 500 µM Pb: Unstained section (Plate 17a, Plate 17b, Plate 17c, Plate 17d and Plate 17e).

Different sizes of Pb electron-dense granules were deposited in various cell regions. The morphology of ribosome, nuclei and endoplasmic reticulum, does not appear to have been adversely affected by the presence of Pb. Damaged membrane of organelles and severe plasmolysis with separations of the plasma membrane from the cell wall were observed in most of the shoot cells (Plate 17a).

In the tracheids, the secondary wall thickenings were lined with scattered small Pb particles, but more were seen within the lignified wall areas, and many fine Pb particles were precipitated in intercellular region. The pit membrane however, had Pb particles scattered throughout (Plate17b).

Some fungus and Pb particles were detected randomly throughout the cytoplasm, while in others, Pb deposits were precipitated in the fungal organelles and outer membrane. Heavy deposits of small Pb particles were scattered in the cytoplasm the tree fern shoot cell (Plate 17c). Heavy deposits of small Pb particles were scattered in the cytoplasm. One very large semi crystalline Pb deposit was observed (Plate 17d). This deposit was confirmed to be Pb using an energy dispersive X-ray analyzer (Figure 14).

Non-Pb treated tree fern (*Cyathea medullaris*): Unstained section of leaf tissue (Plate 18).

The leaf tissue, chloroplasts with grana, stroma thylakoids, and small plastoglobuli were found within the large regular central vacuolated cells. Mitochondria were observed in the cytoplasm. The cell wall was normal in appearance. No Pb deposits were found in control cells (Plate 18).

Leaf tissue from the treatment with 250 µM Pb: Unstained section (Plate 19).

The epidermal leaf cells contained large central vacuoles. Starch grains of different sizes appeared inside the chloroplasts. The thickness of the cell walls increased noticeably, smaller Pb deposits could also be seen adjacent and aligned with the cell wall (Plate 19).

Leaf tissue from the treatment with 500 µM Pb: Unstained section (Plate 20).

Pb particles were clearly visible in cytoplasm and adjacent to the thick cell walls. The amount of Pb precipitate increased compared to the 250 µM Pb treated leaves. No Pb particles were found in the cell organelles or cytoplasm at a higher magnification (Plate 20).

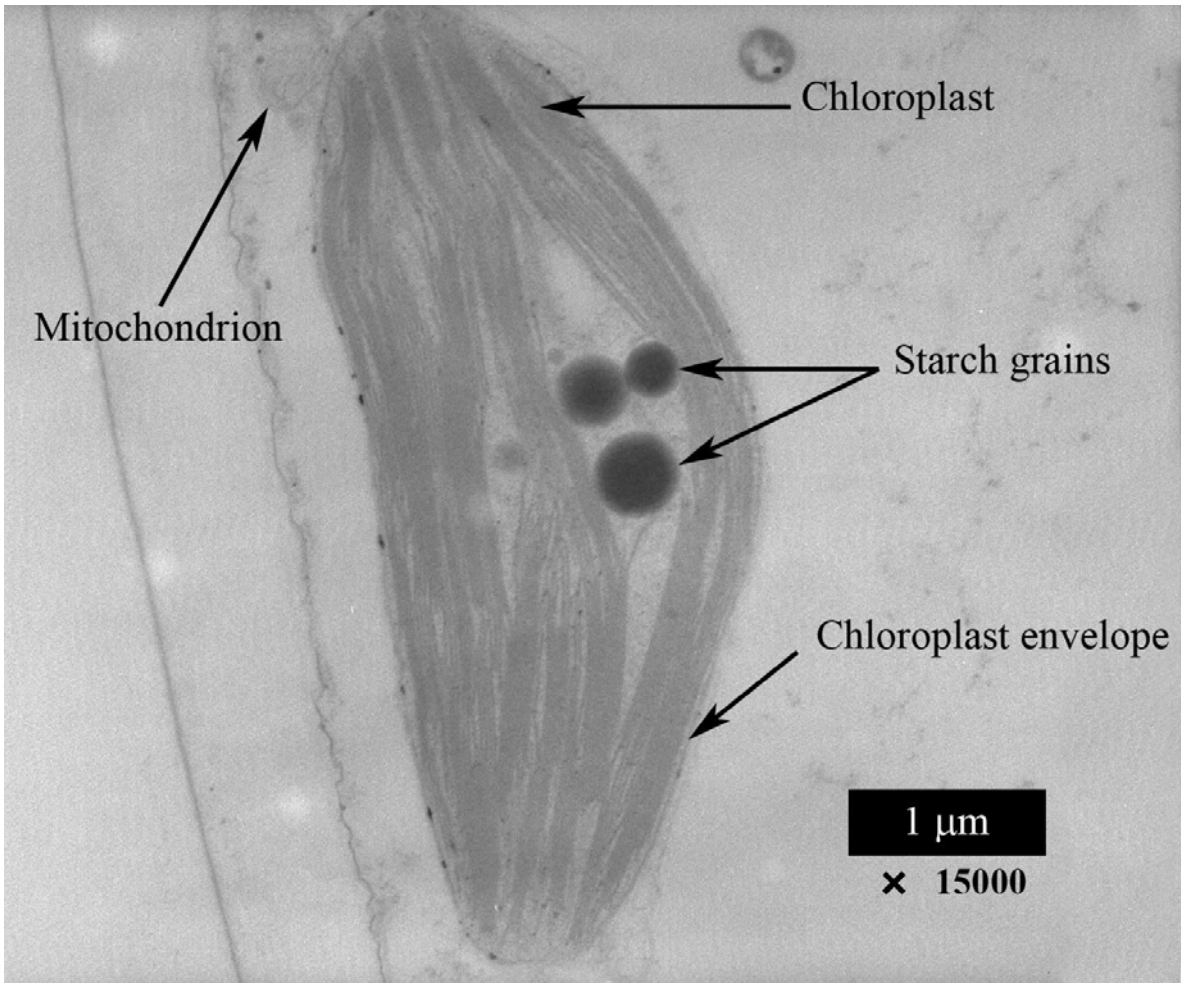

Plate 5:- Transmission electron micrograph (15,000 X magnifications) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte incubated in water only (control) for 3 days.

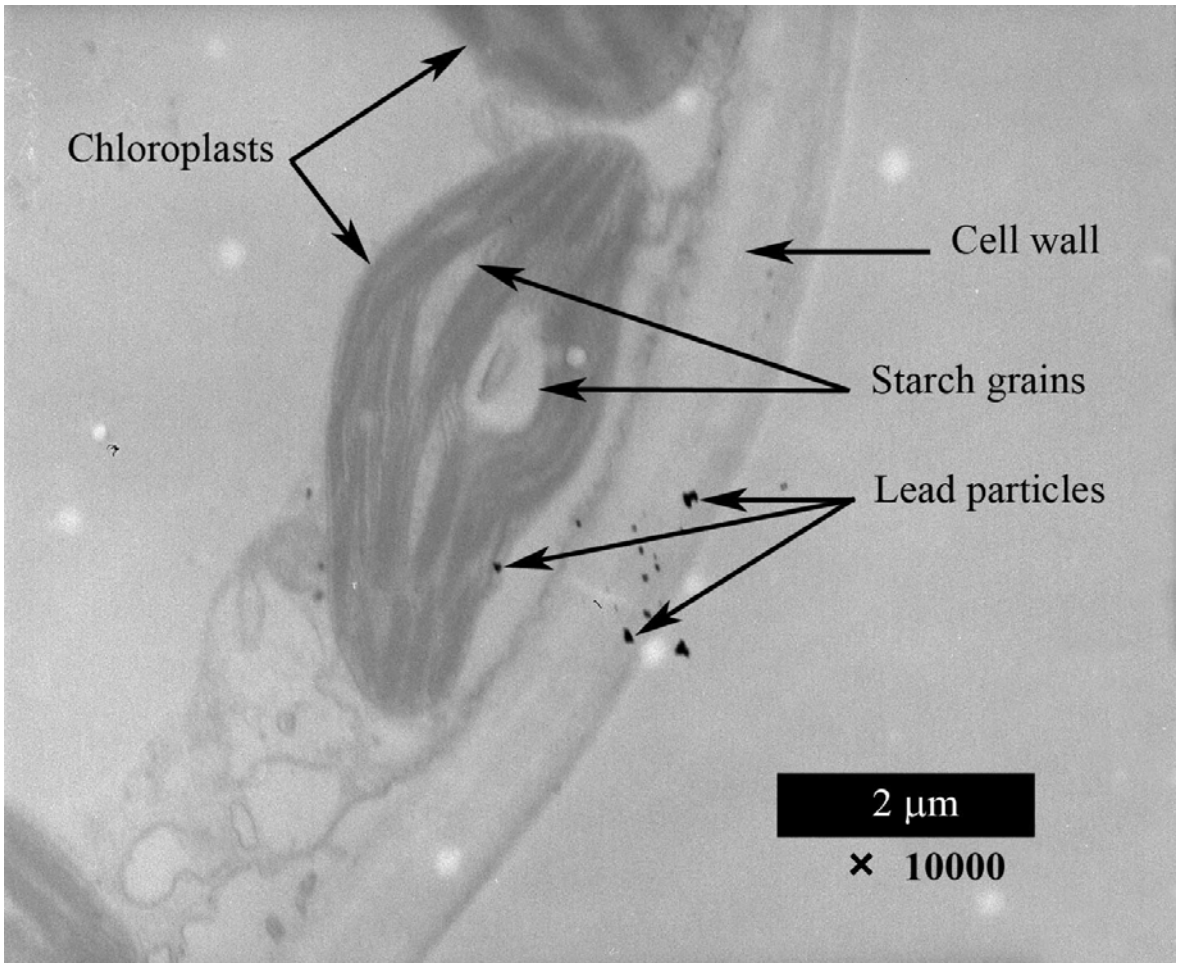

Plate 6:- Transmission electron micrograph (10,000X magnification) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 250 µM of $Pb(NO_3)_2$ for 3 days.

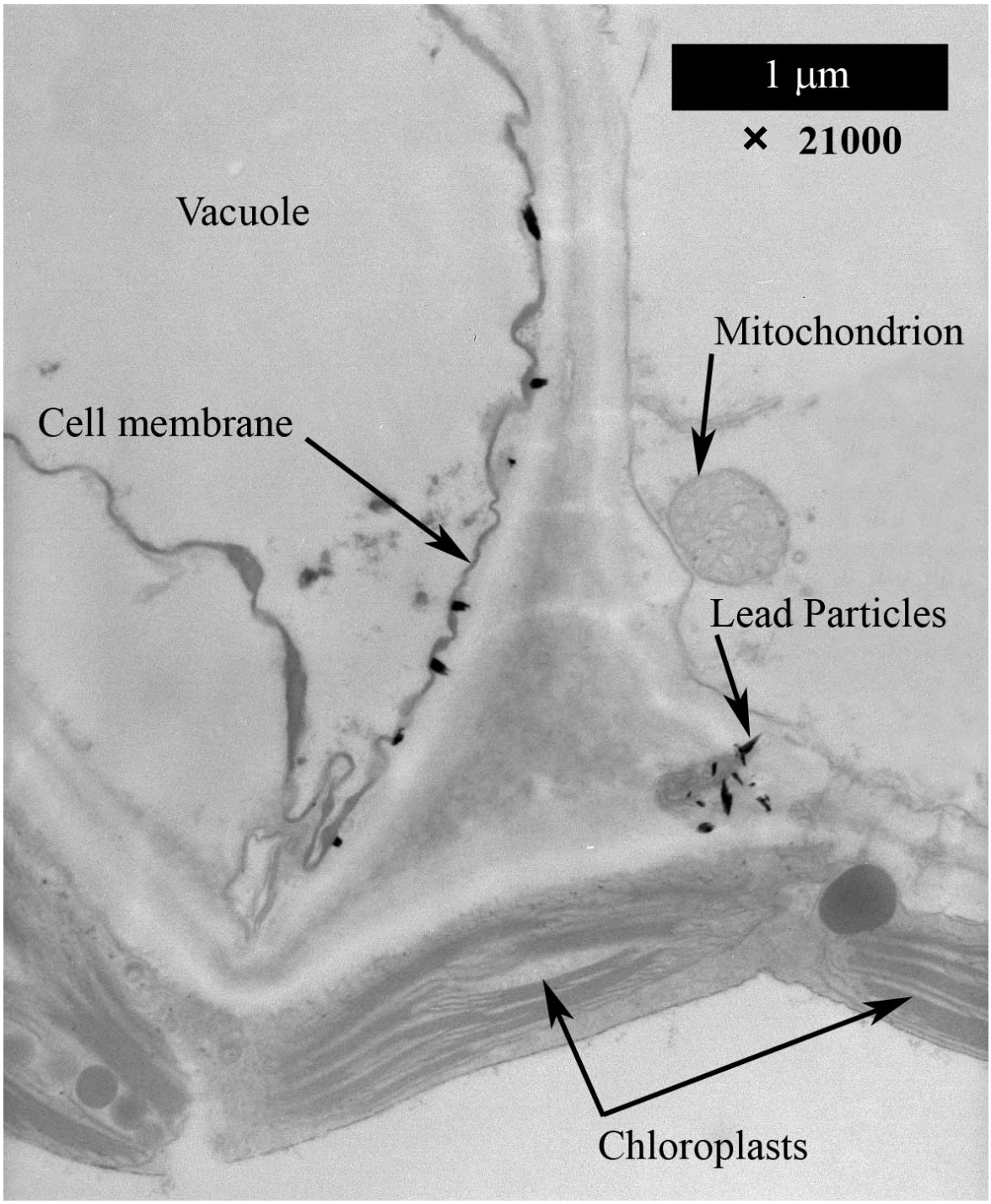

Plate 7a:- Transmission electron micrograph (21,000X magnification) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 500 µM $Pb(NO_3)_2$ for 3 days.

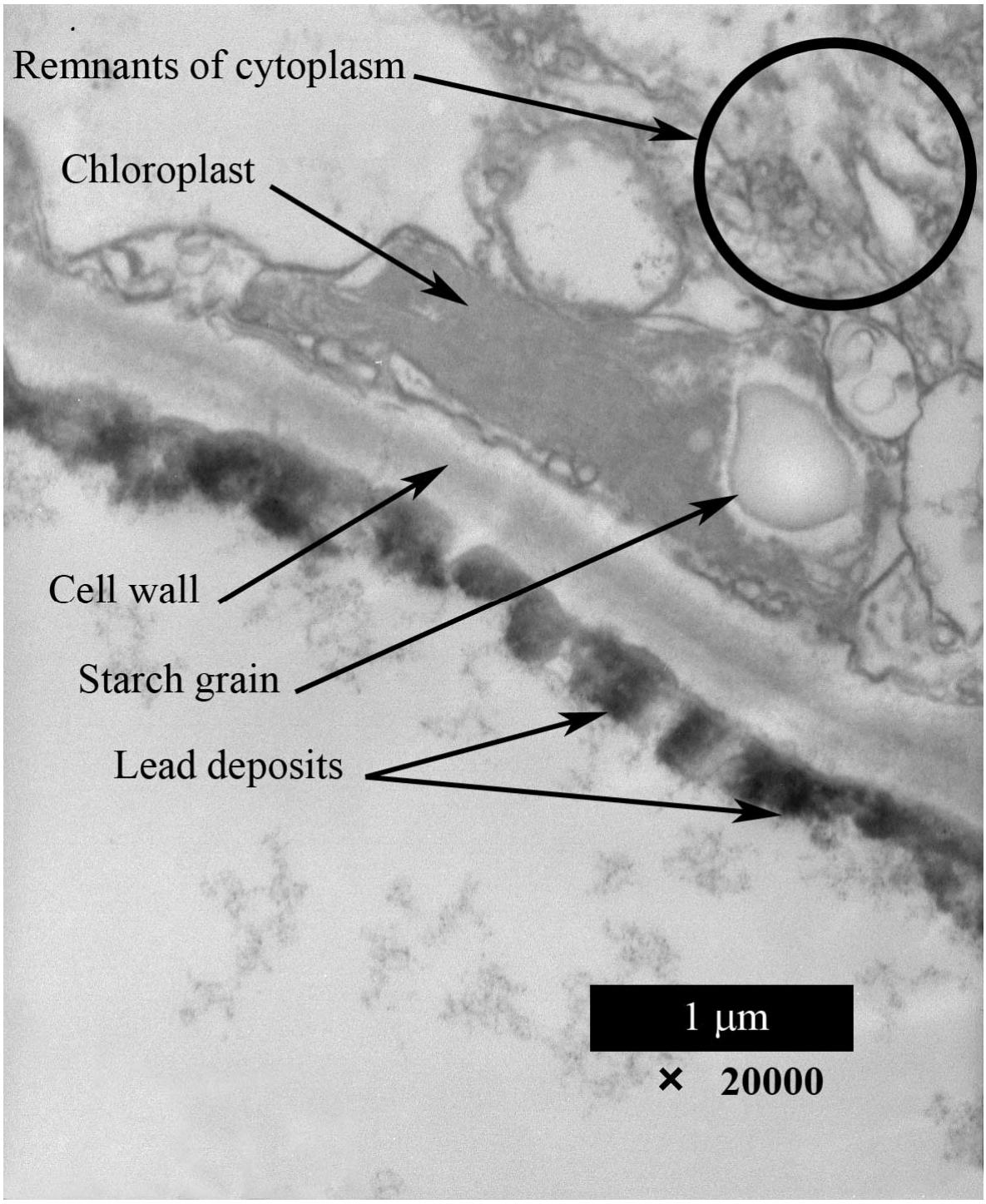

Plate 7b:- Transmission electron micrograph (20,000X magnification) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 500 µM of $Pb(NO_3)_2$ for 3 days.

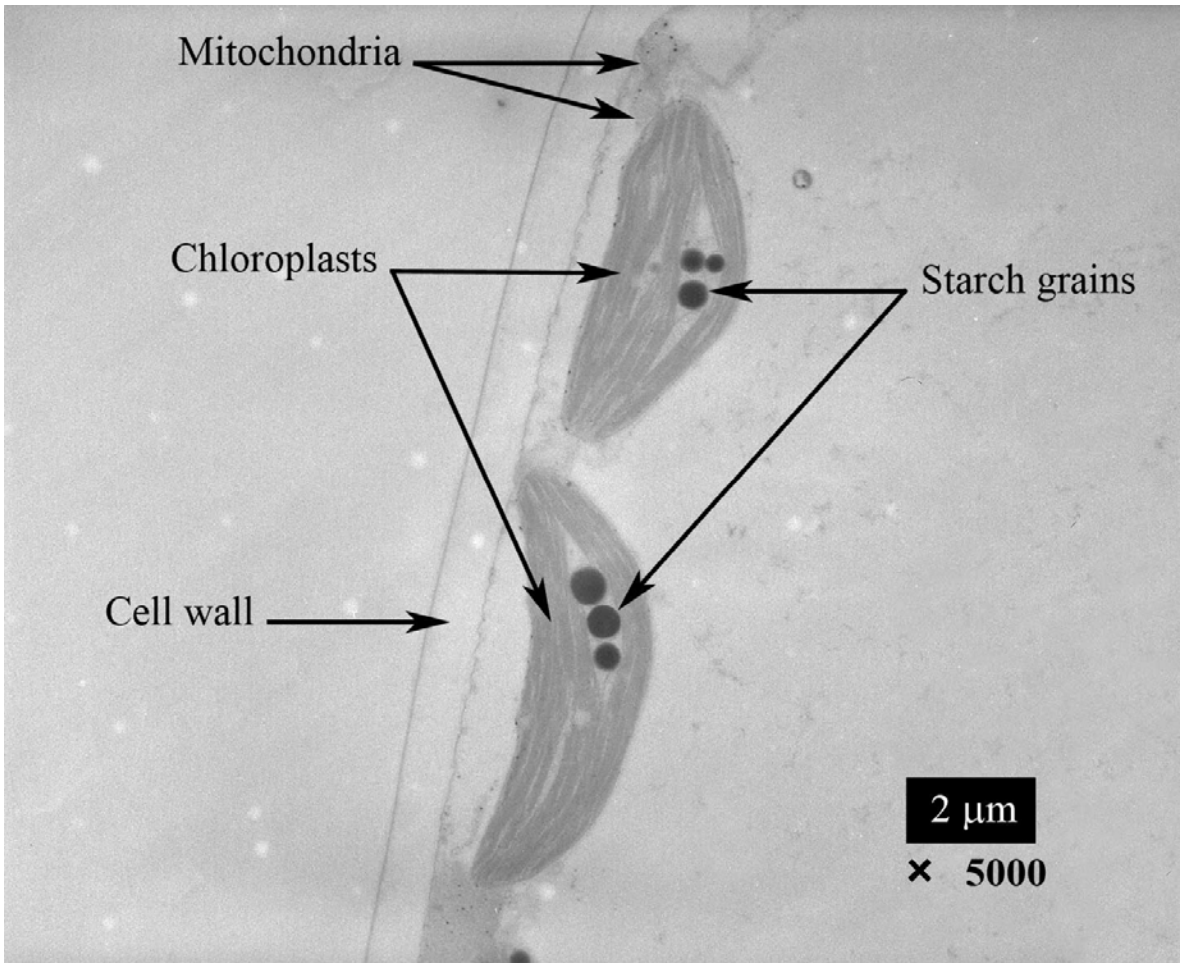

Plate 8:- Transmission electron micrograph (5,000X magnification) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte incubated in water for 21 days.

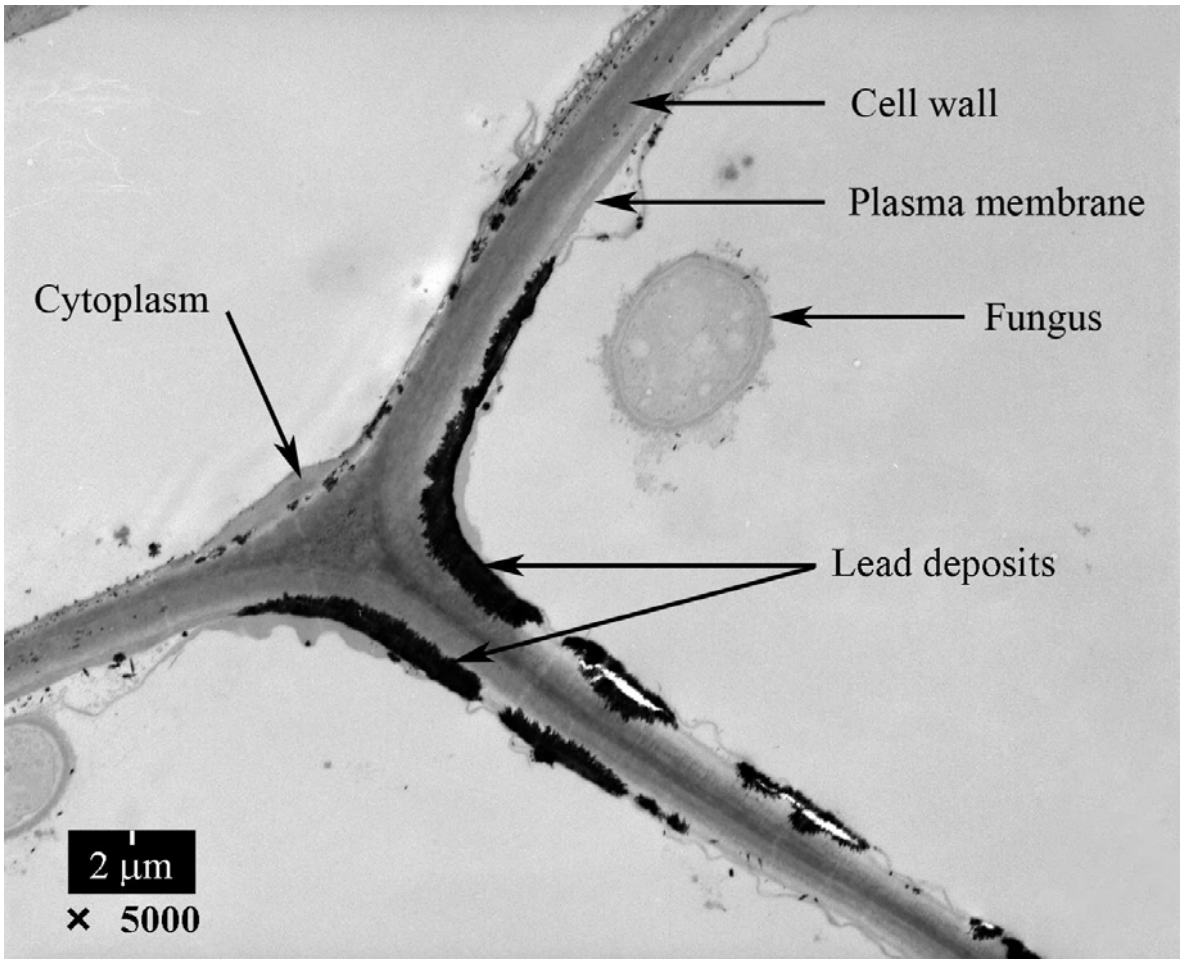

Plate 9:- Transmission electron micrograph (5,000X magnification) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 250 µM of $Pb(NO_3)_2$ for 21 days.

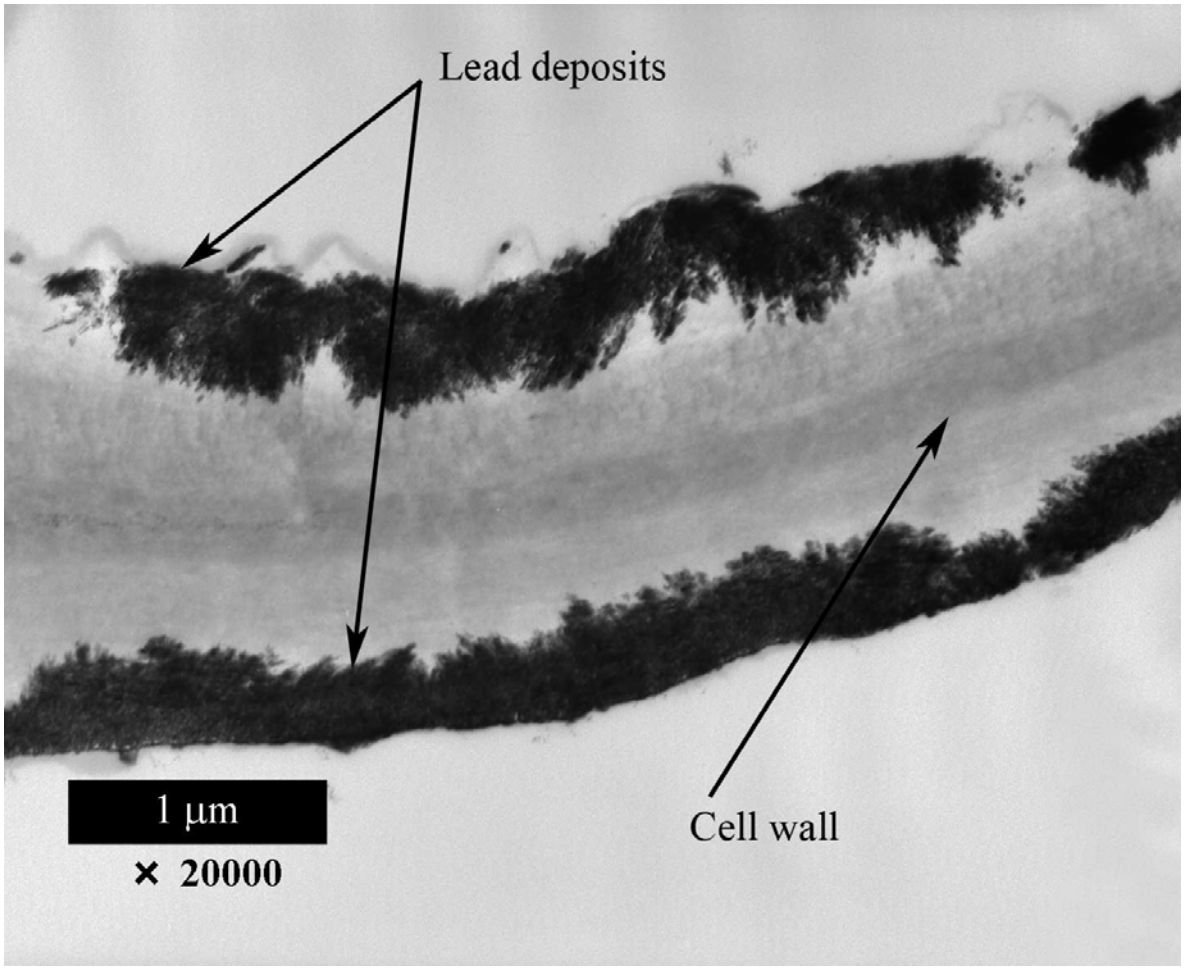

Plate 10a:- Transmission electron micrograph (20,000X magnification) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

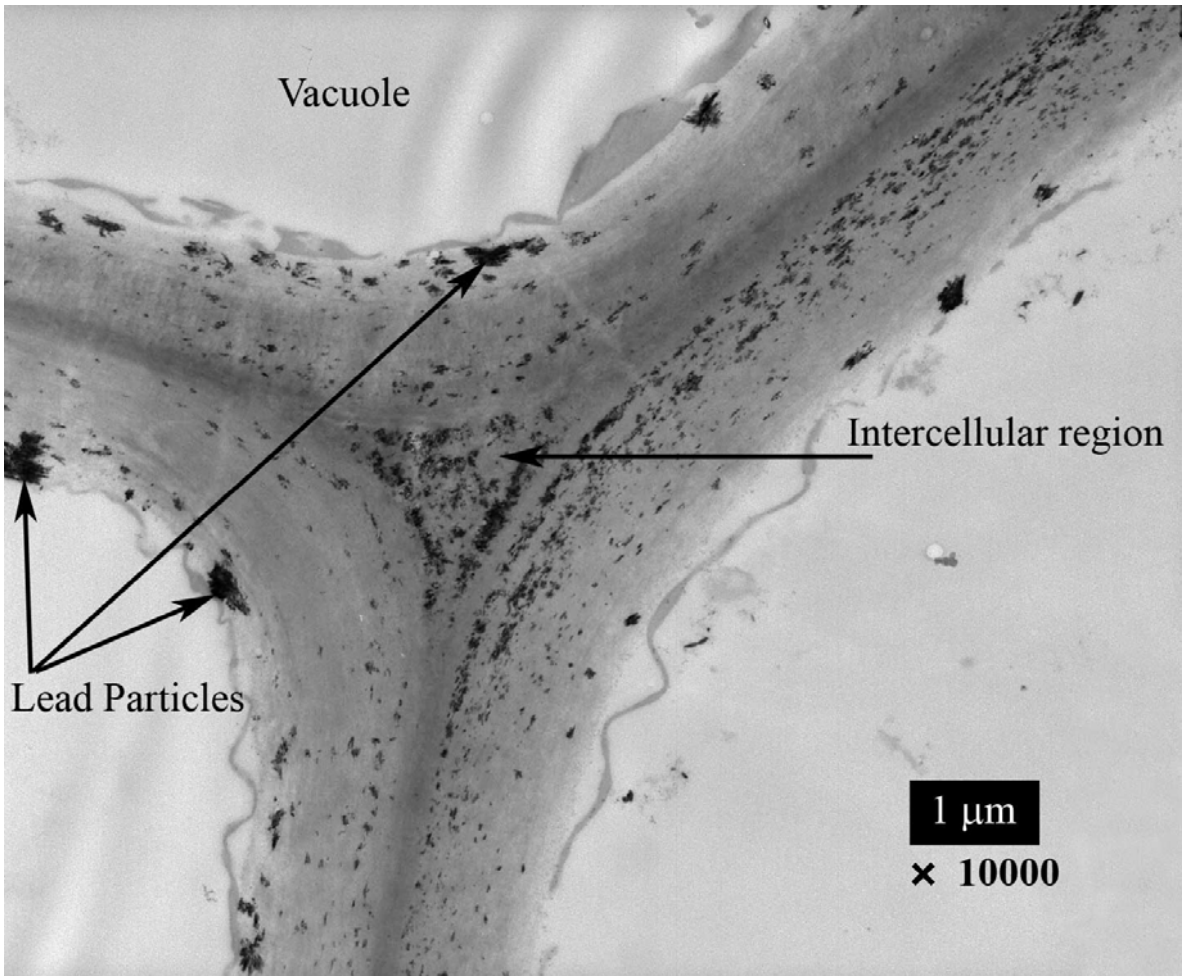

Plate 10b:- Transmission electron micrograph (10,000X magnification) of an unstained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

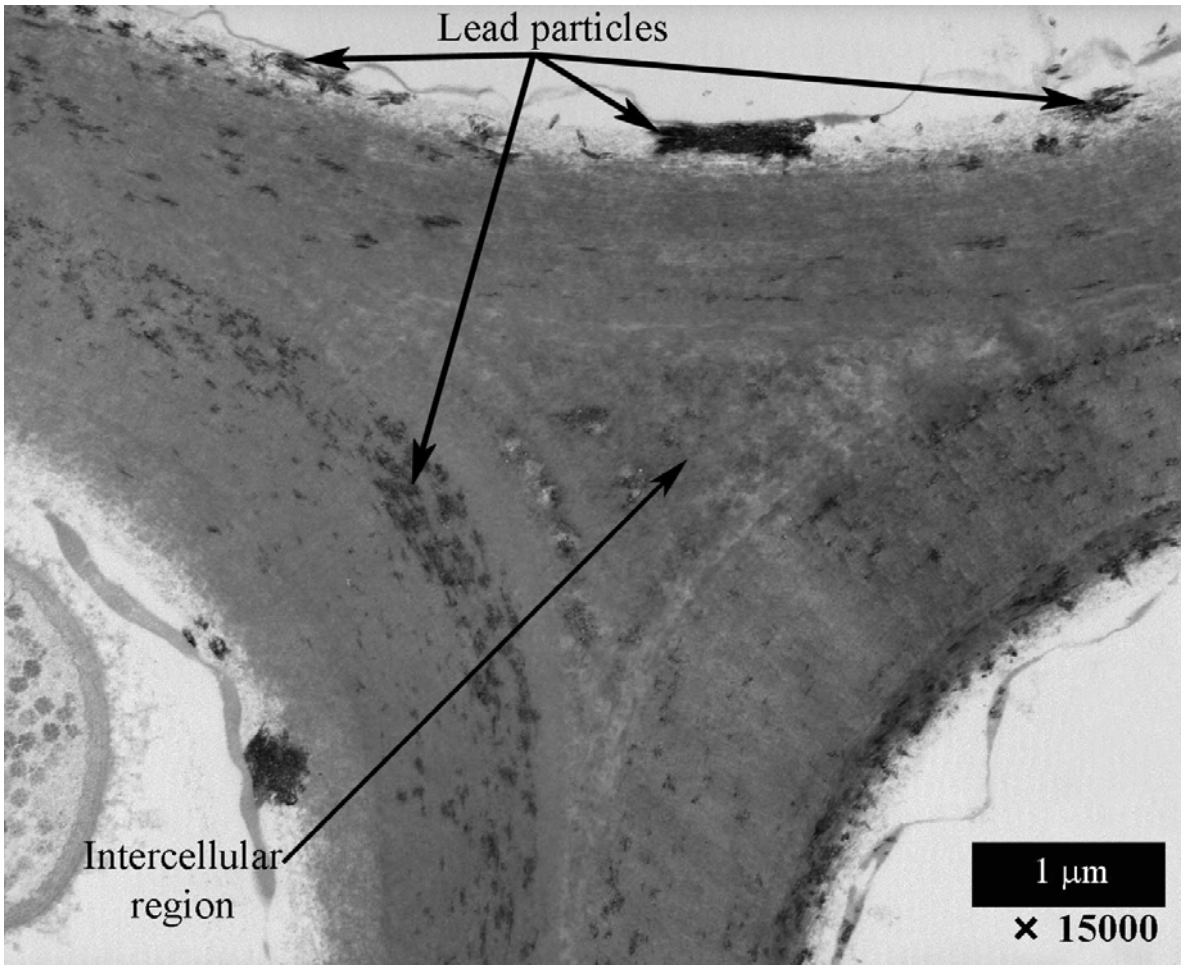

Plate 11a:- Transmission electron micrograph (15,000X magnification) of stained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

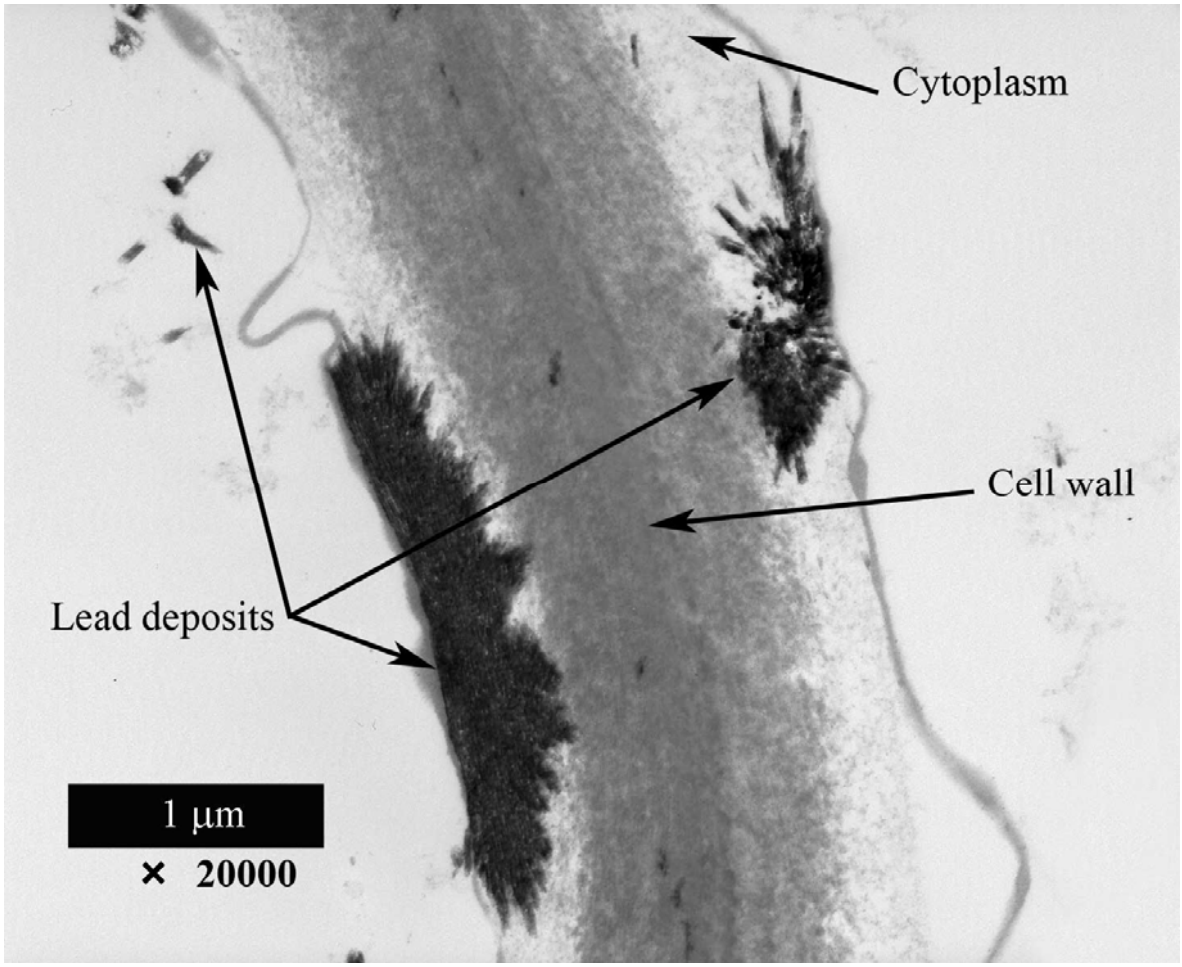

Plate 11b:- Transmission electron micrograph (20,000X magnification) of stained ultrathin section of tree fern *(Cyathea medullaris)* gametophyte treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

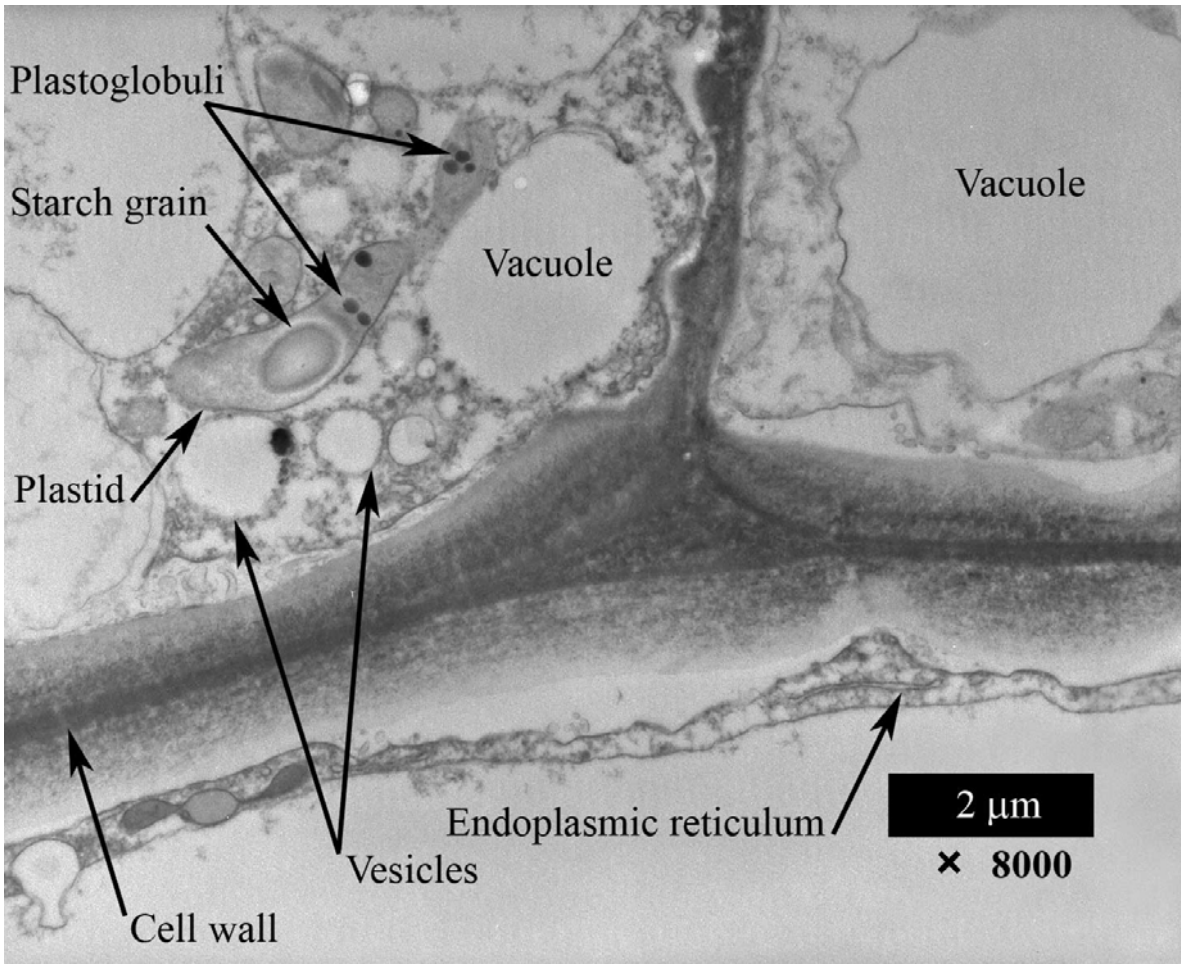

Plate 12:- Transmission electron micrograph (8, 000X magnification) of an unstained ultrathin section of a tree fern *(Cyathea medullaris)* root without Pb treatment (control) for 21 days.

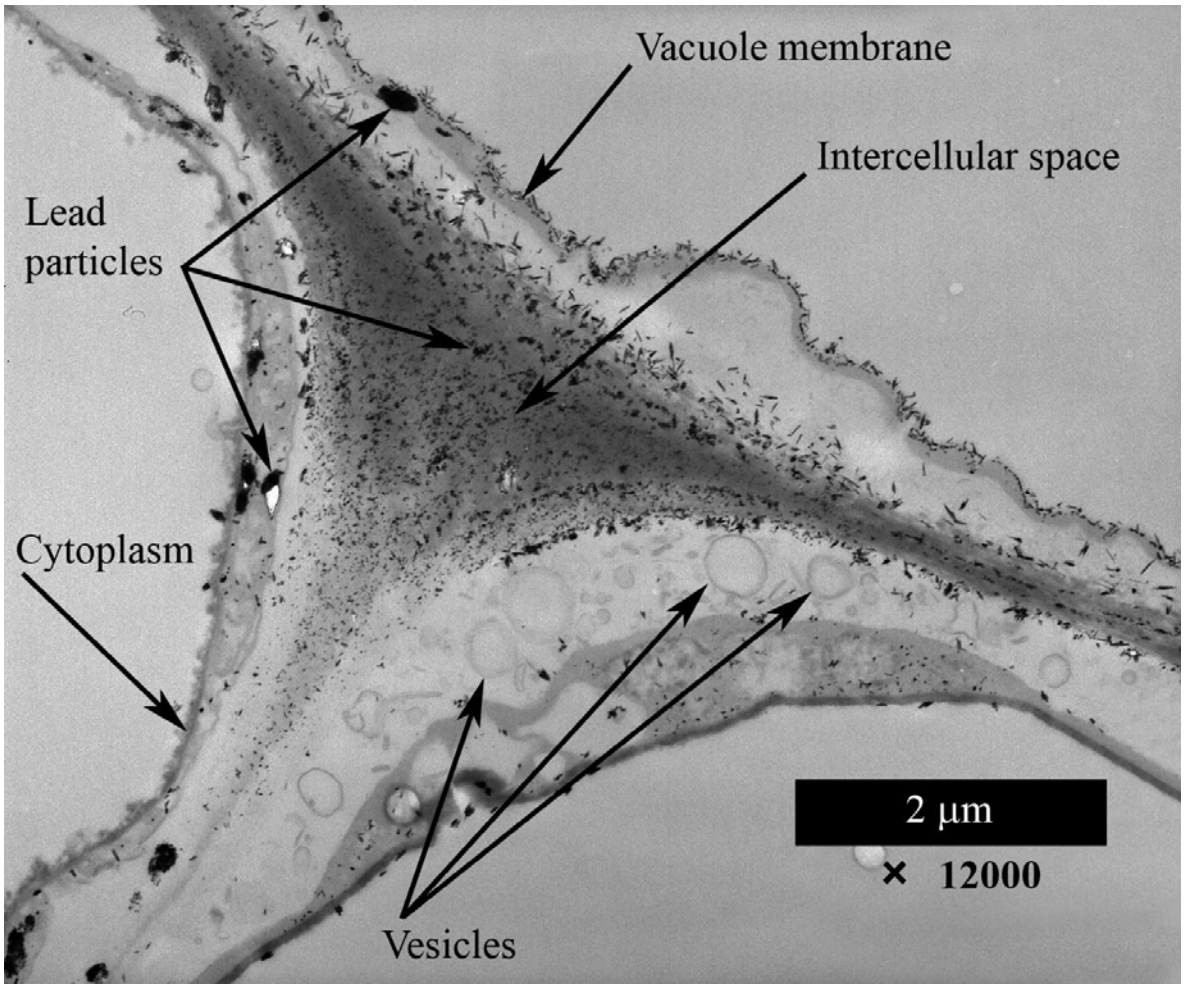

Plate 13a:- Transmission electron micrograph (12,000X magnification) of an unstained ultrathin section of a root from a tree fern *(Cyathea medullaris)* plant treated with 250 µM of $Pb(NO_3)_2$ for 21 days.

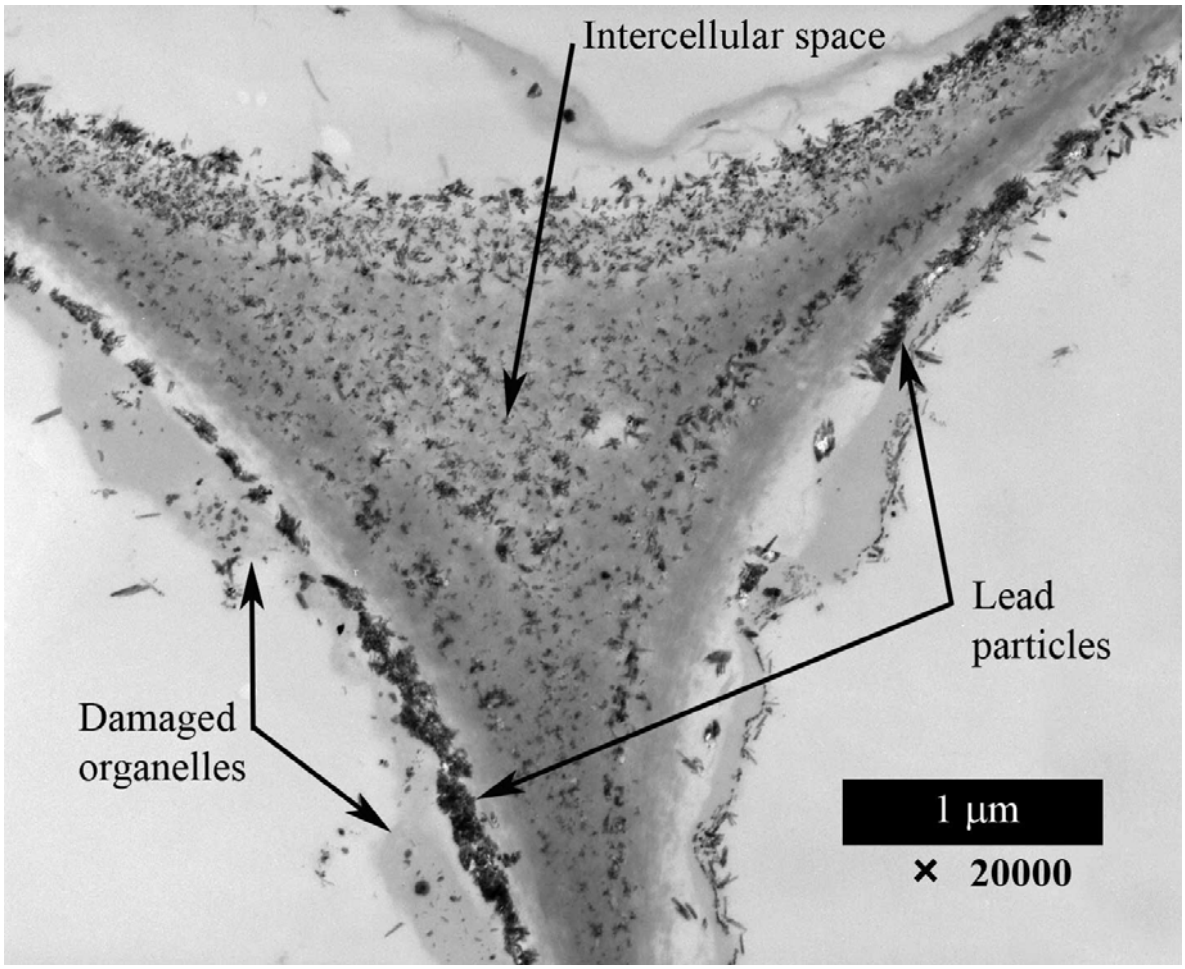

Plate 13b:- Transmission electron micrograph (20,000X magnification) of an unstained ultrathin section of a root from a tree fern *(Cyathea medullaris)* plant treated with 250 µM of $Pb(NO_3)_2$ for 21 days.

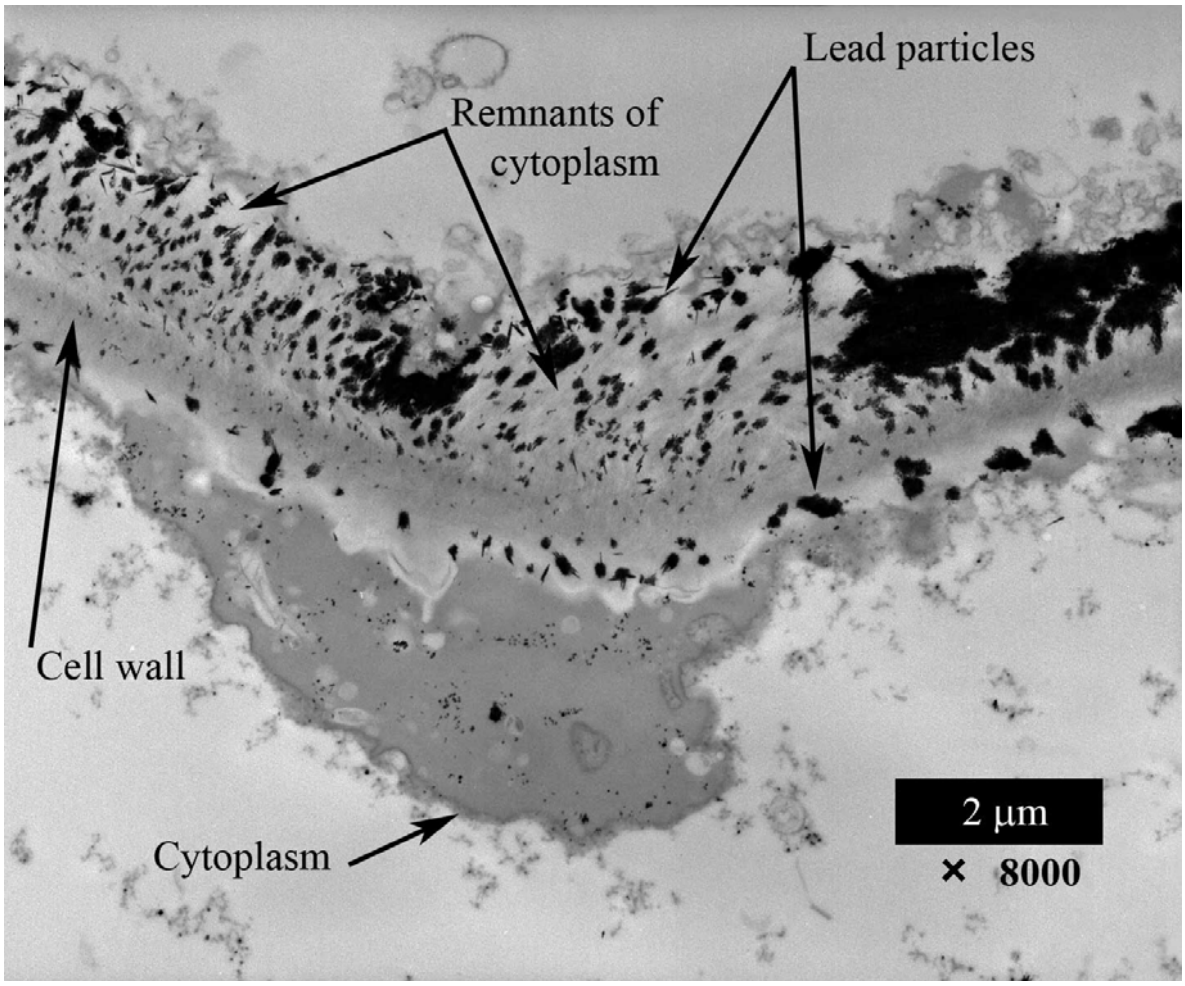

Plate 14a:- Transmission electron micrograph (8,000X magnification) of an unstained ultrathin section of a root from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

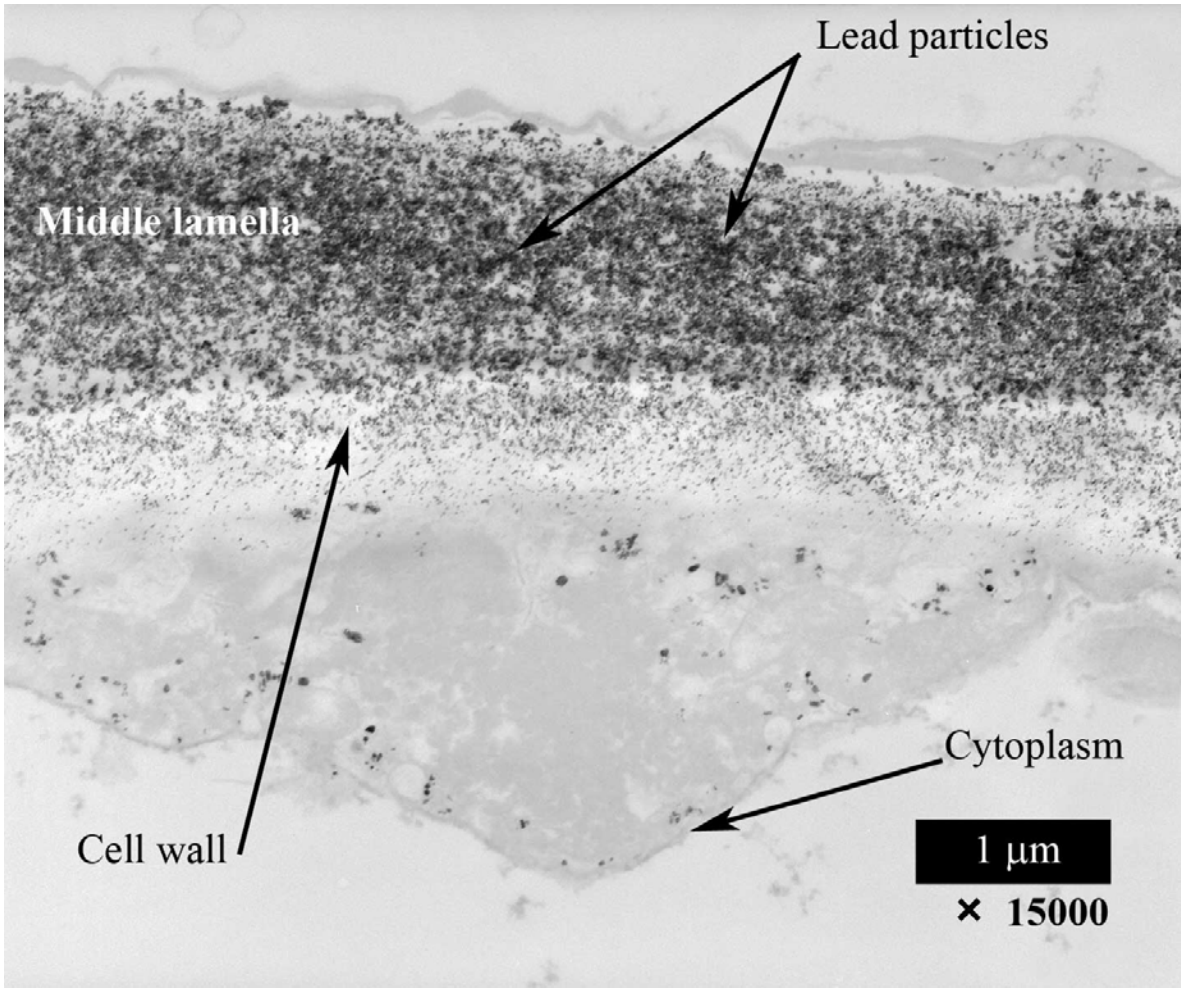

Plate 14b:- Transmission electron micrograph (15,000X magnification) of an unstained ultrathin section of a root from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

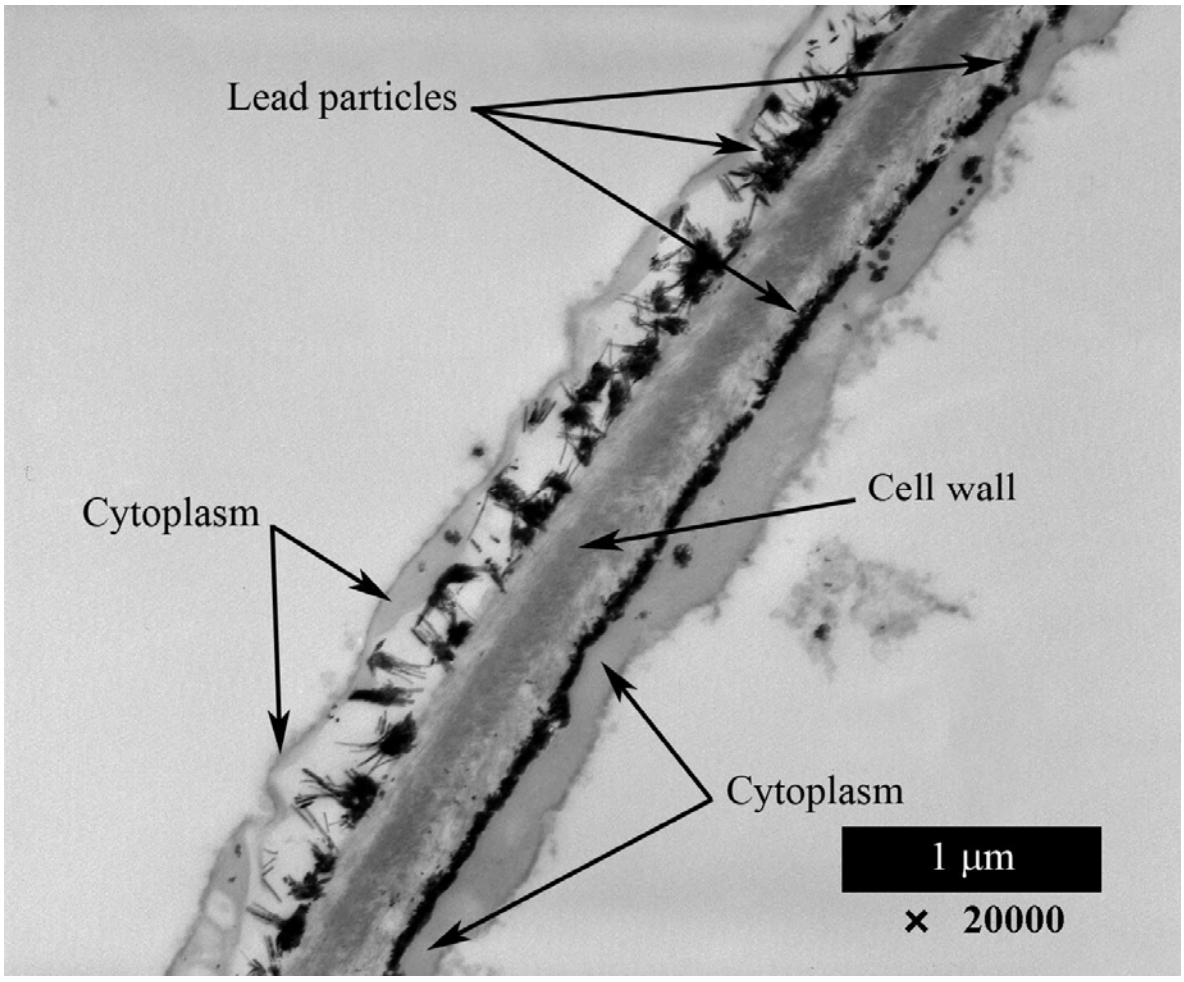

Plate 14c:- Transmission electron micrograph (20,000X magnification) of an unstained ultrathin section of a root from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

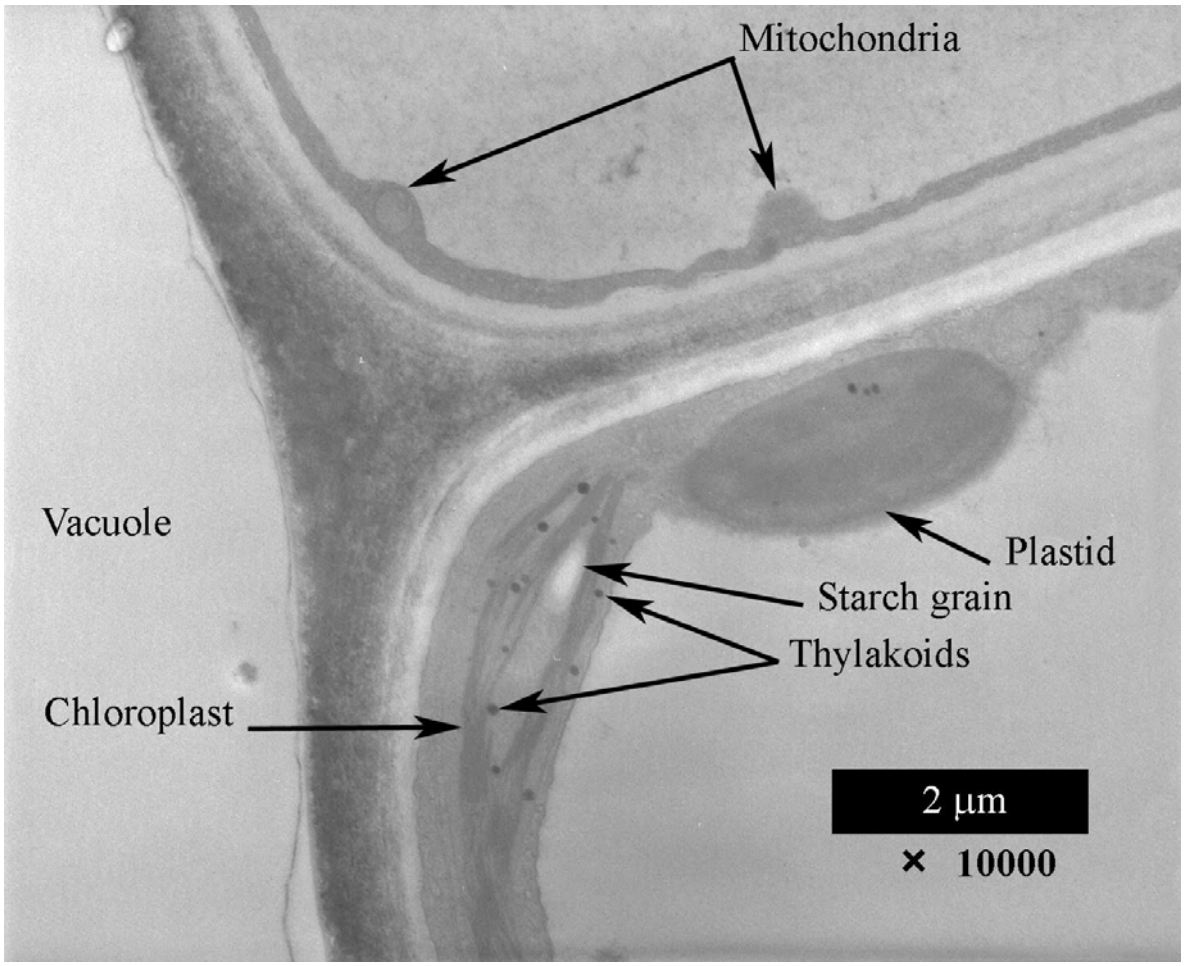

Plate 15:- Transmission electron micrograph (10,000X magnification) of an unstained ultrathin section of a tree fern *(Cyathea medullaris)* shoot grown in water without Pb for 21 days.

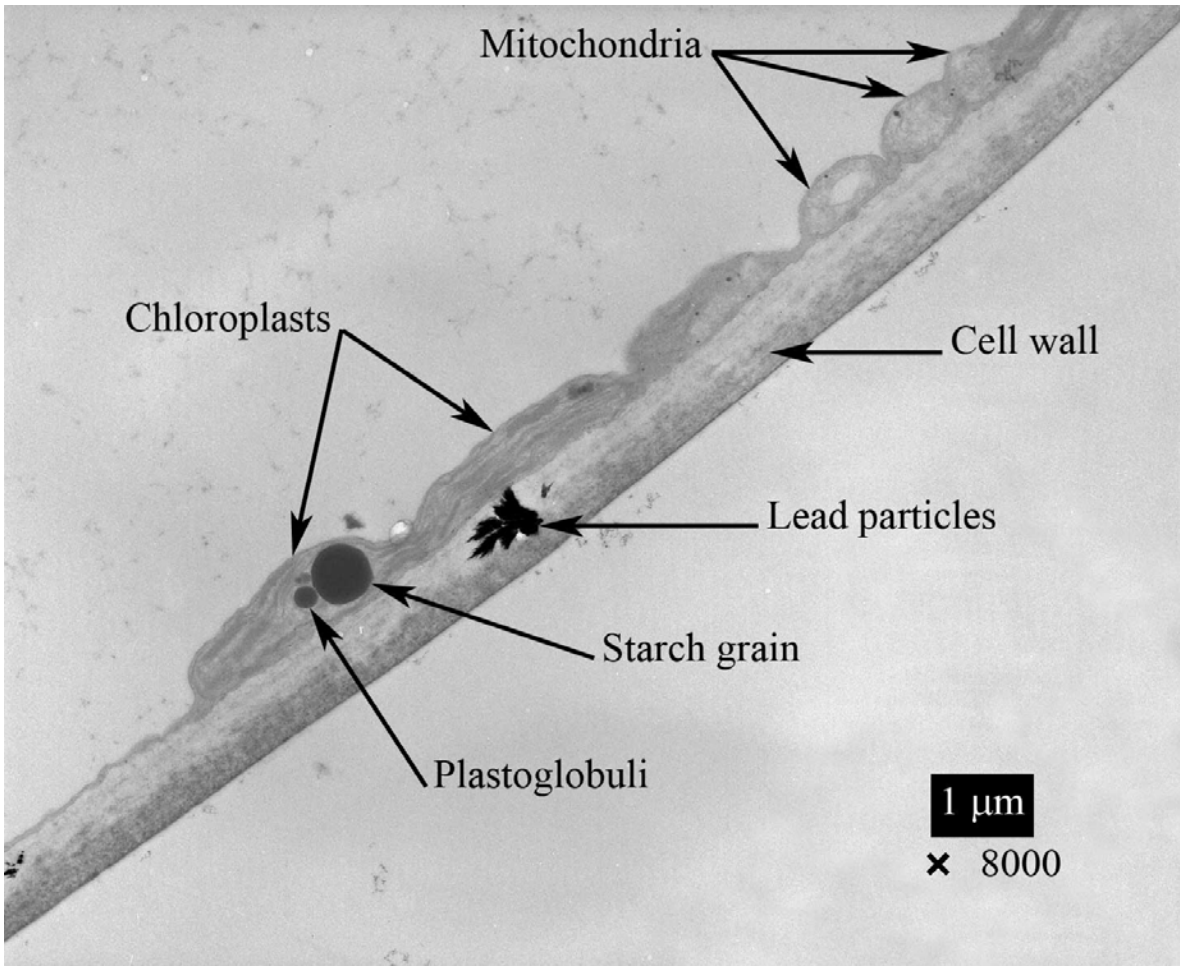

Plate 16a:- Transmission electron micrograph (8,000X magnification) of an unstained ultrathin section of a shoot from a tree fern *(Cyathea medullaris)* plant treated with 250 µM of $Pb(NO_3)_2$ for 21 days.

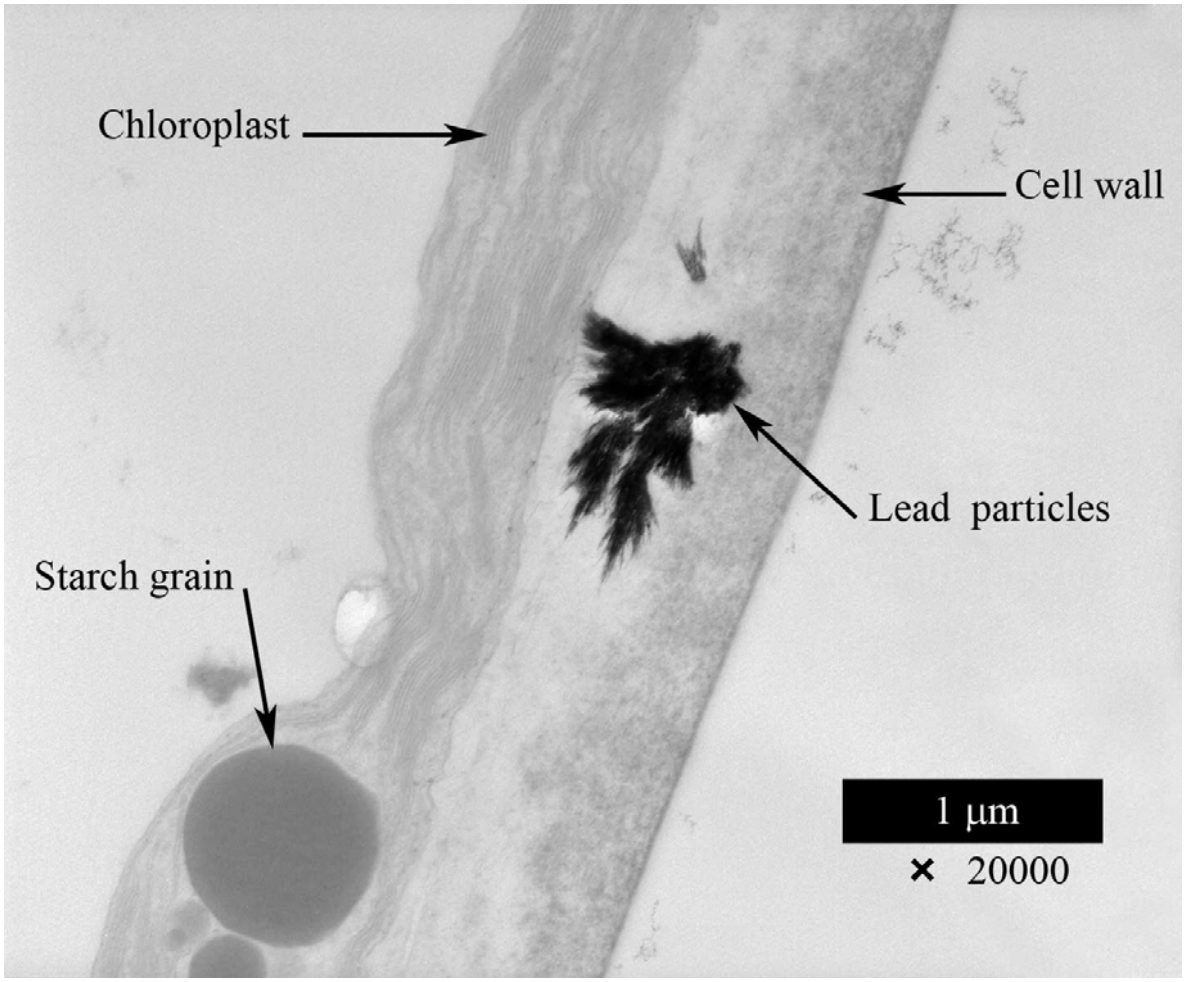

Plate 16b:- Transmission electron micrograph (20,000X magnification) of an unstained ultrathin section of a shoot from a tree fern *(Cyathea medullaris)* plant treated with 250 µM of $Pb(NO_3)_2$ for 21 days.

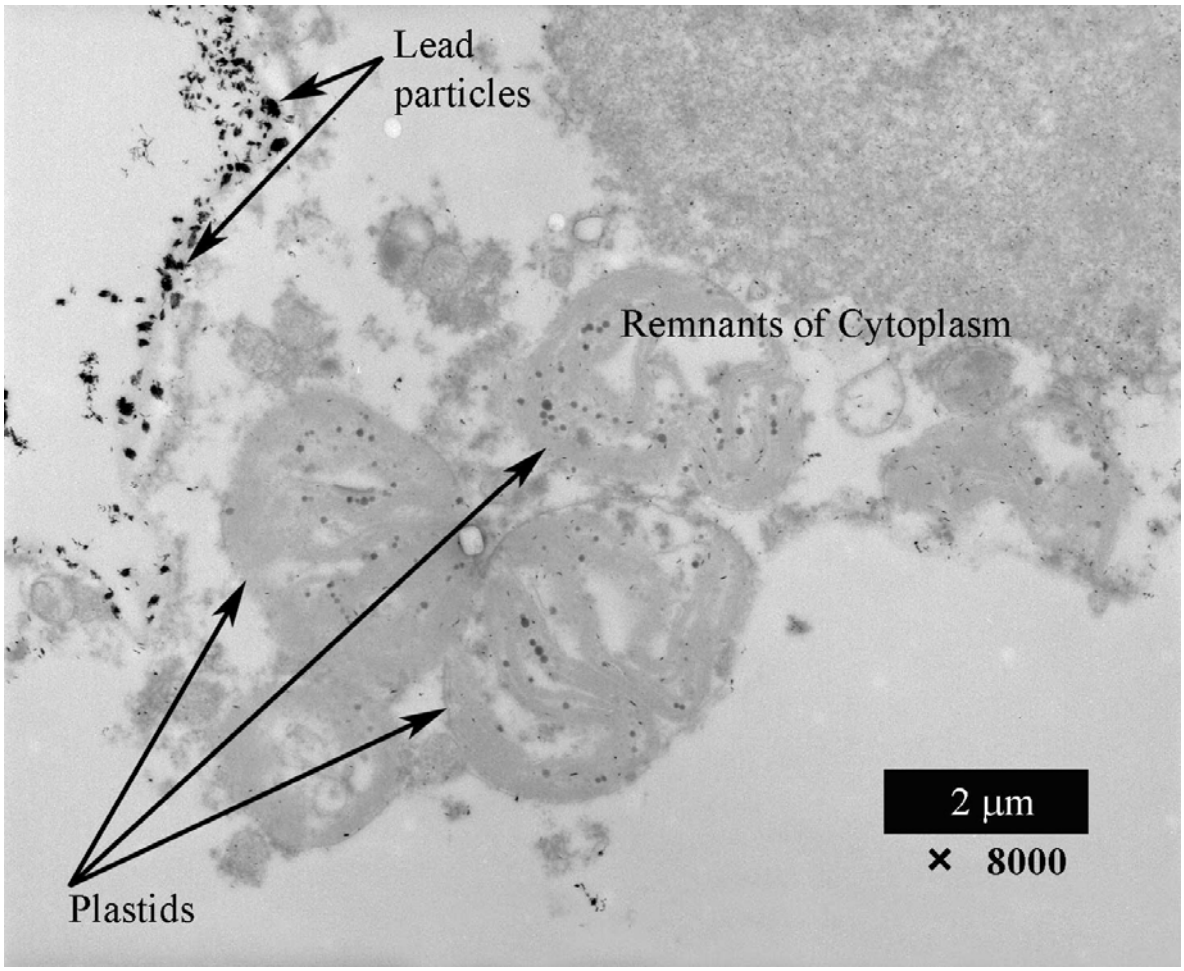

Plate 17a:-Transmission electron micrograph (8,000X magnification) of an unstained ultrathin section of a shoot from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

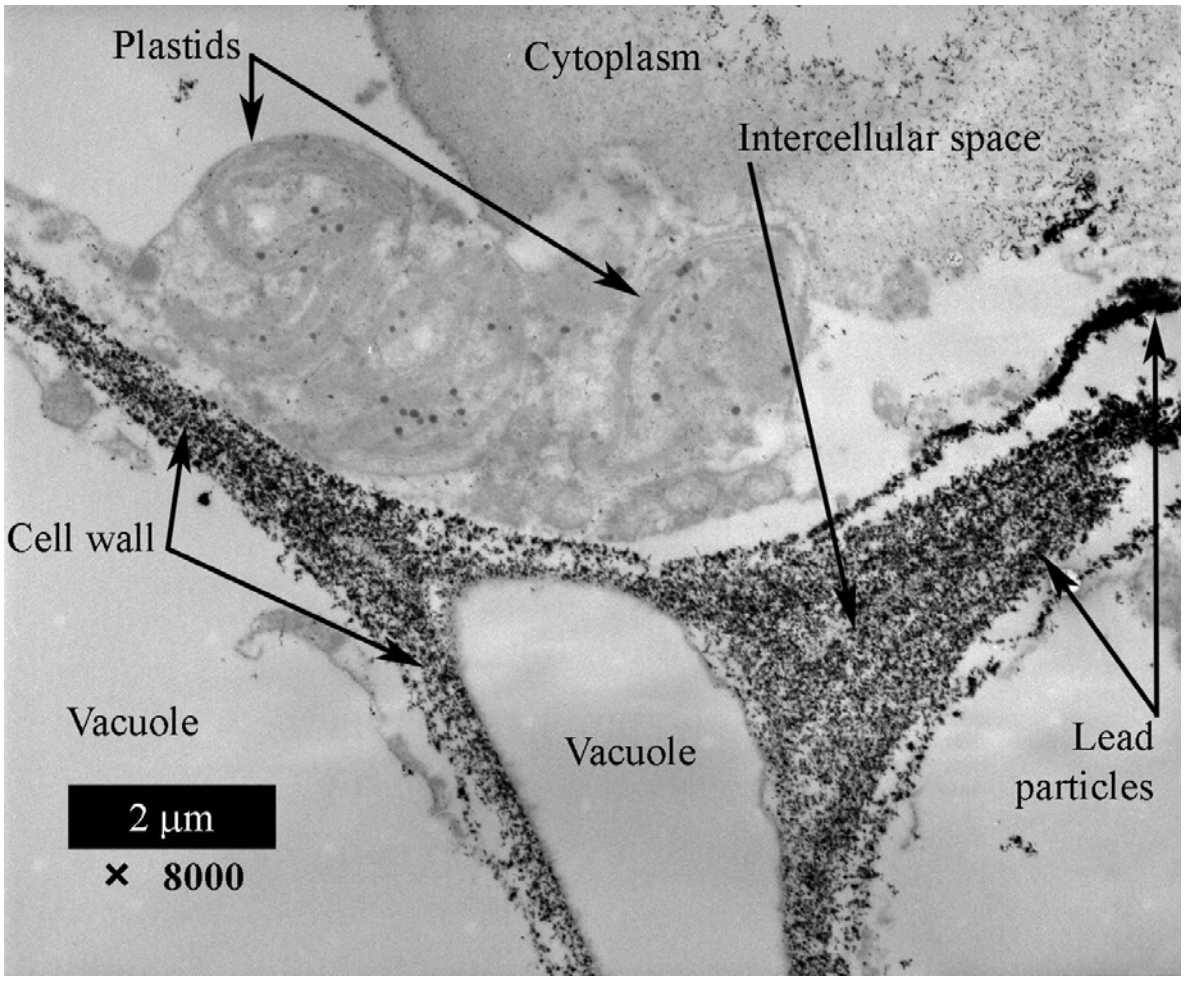

Plate 17b:-Transmission electron micrograph (8,000X magnification) of an unstained ultrathin section of a shoot from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

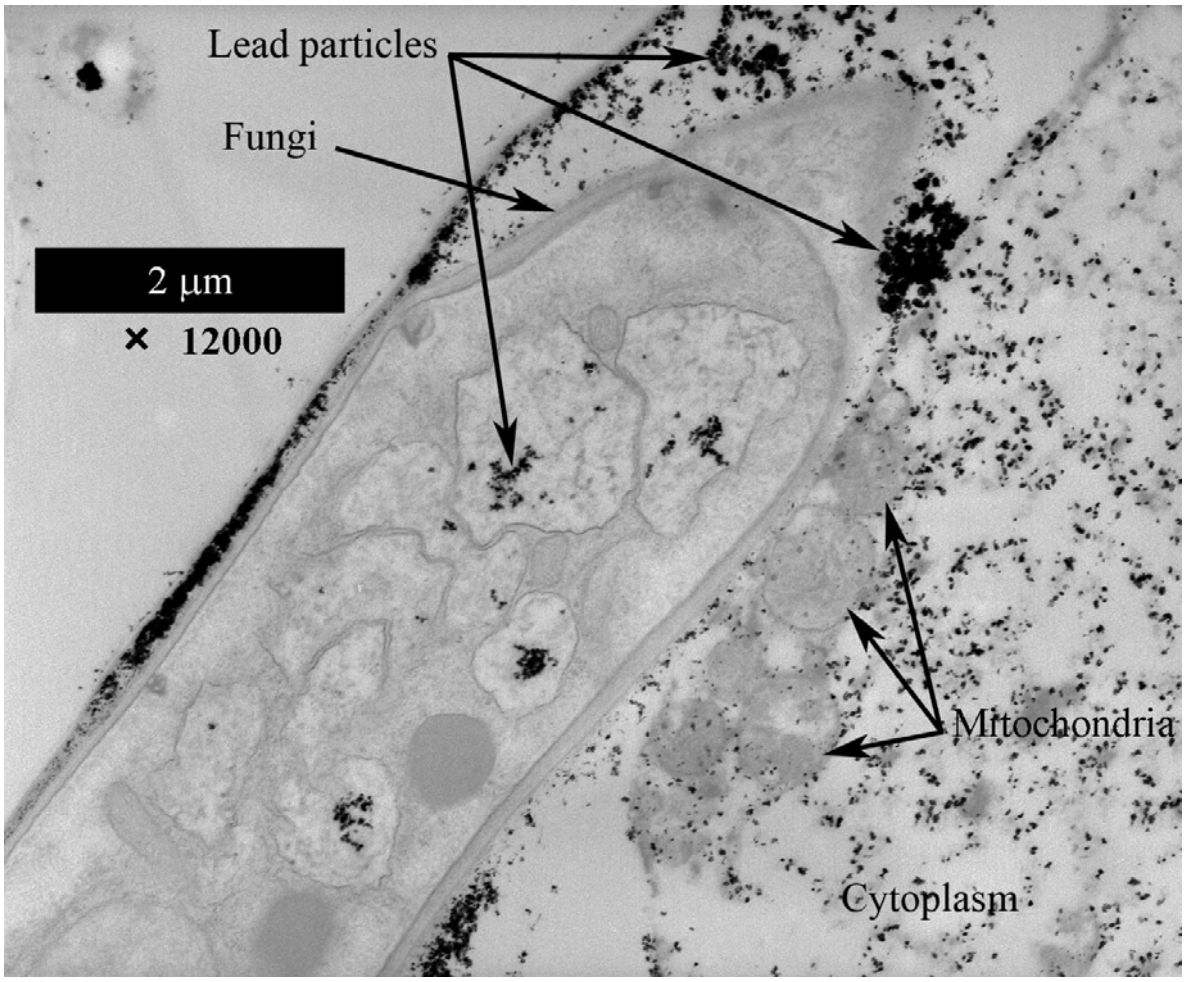

Plate 17c:-Transmission electron micrograph (12,000X magnification) of an unstained ultrathin section of a shoot from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

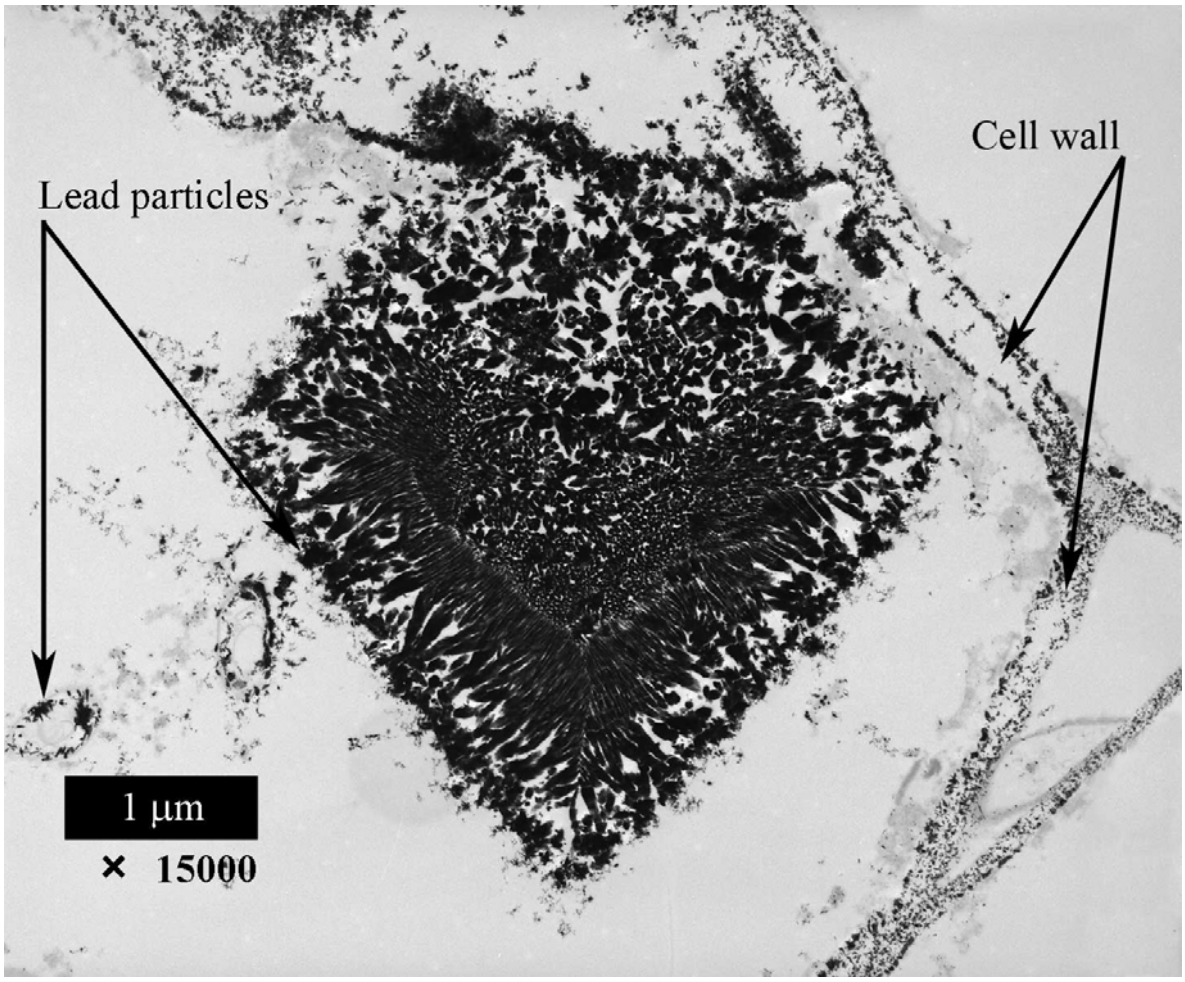

Plate 17d:- Transmission electron micrograph (15,000X magnification) of an unstained ultrathin section of a shoot from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

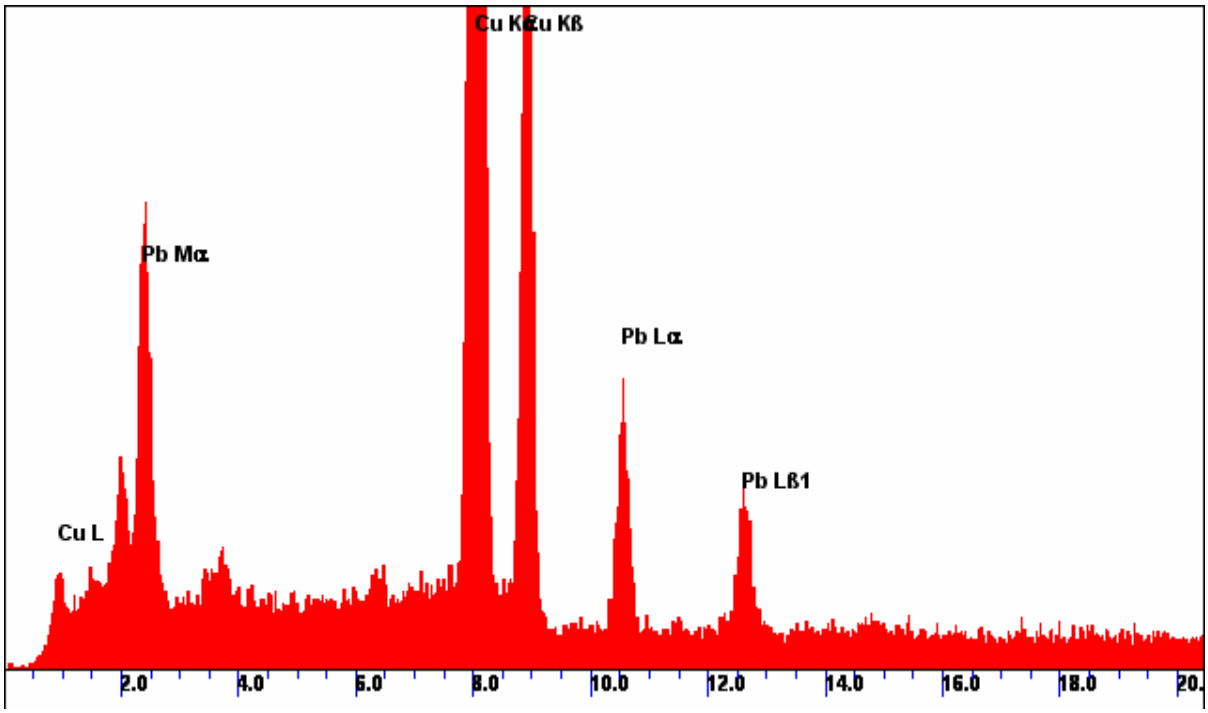

Figure 14:– The crystalline deposit seen in plate 17e was confirmed to be Pb using an analytical TEM with energy dispersive X-ray analyzer.

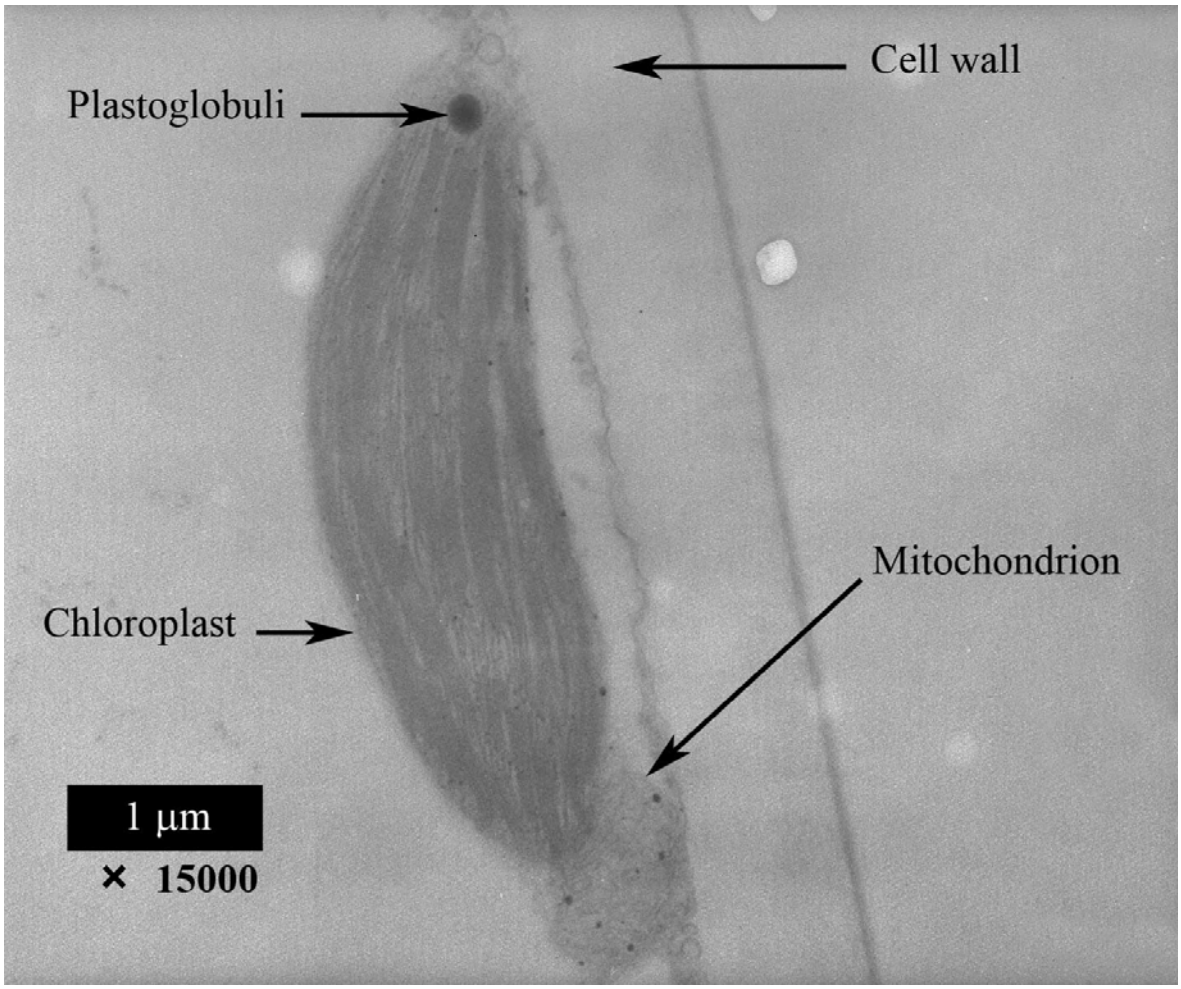

Plate 18:- Transmission electron micrograph (15, 000X magnification) of an unstained ultrathin section of a tree fern *(Cyathea medullaris)* leaf without Pb treatment (control) for 21 days.

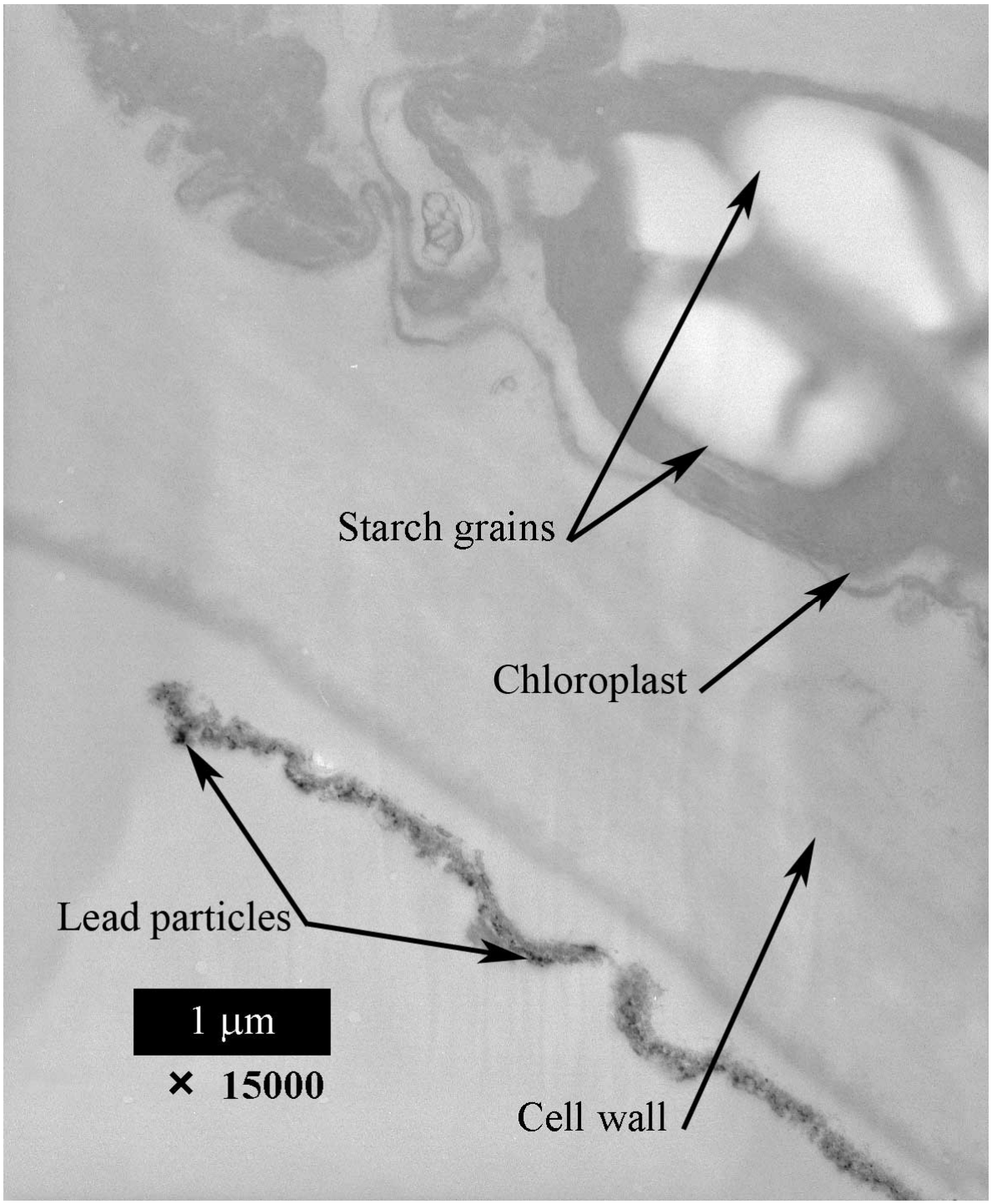

Plate 19:- Transmission electron micrograph (15,000X magnification) of an unstained, ultrathin section of a leaf from a tree fern *(Cyathea medullaris)* plant treated with 250 µM of $Pb(NO_3)_2$ for 21 days.

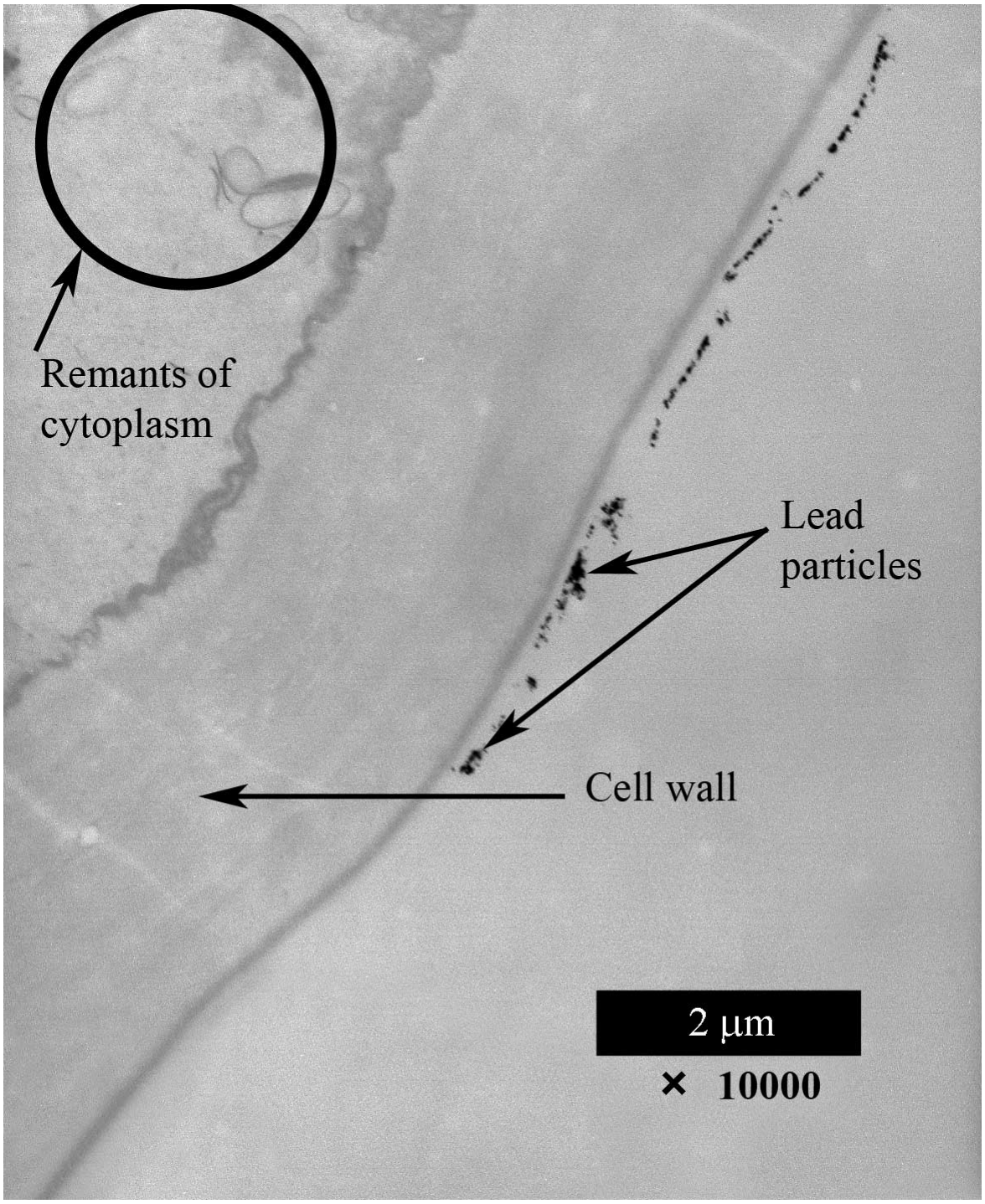

Plate 20:- Transmission electron micrograph (10,000X magnification) of an unstained, ultrathin section of a leaf from a tree fern *(Cyathea medullaris)* plant treated with 500 µM of $Pb(NO_3)_2$ for 21 days.

CHAPTER 4.0

DISCUSSION

4.1 Pb uptake by black tree fern gametophytes

There are some ferns that accumulate exceptionally large amounts of metals in their tissues (Baker and Brooks 1989; Wenzel and Jockwer, 1999).The gametophytes of *Athyrium yokscense* is one of the plants that has been demonstrated to accumulate and resist Pb toxicity (Honjo *et al*., 1980). Likewise, in *Pteris vittata*, a well-known arsenic hyperaccumulator (Ma *et al*., 2001; Wang *et al.*, 2002), its gametophytes were found to be highly defiant to arsenic toxicity. Therefore, it seems likely that fern gametophytes might have the unusual ability to adapt to extremely harsh external environment.

The presence of Pb in the gametophytes of *Cyathea medullaris* was visually detectable by the colour of their ashes, it ranged from yellowish orange through to red colour indicating a huge presence of the Pb. This may indicate that Pb oxidation occurrence in the gametophyte ash and it was converted to red Pb (trilead tetreoxide). In addition, this compound has a high molecular weight due to more Pb molecules in its structure.

Pb concentration in plants commonly does not normally exceed 5 mg/kg dry weight. Gametophytes of the New Zealand black tree fern *(Cyathea medullaris)* accumulated an unusually large amount of Pb. For example, more than 18,000 mg Pb accumulated per Kg dry weight after treatment with 250 µM $Pb(NO_3)_2$ for 21 days (see Figure 8).

It is generally believed that the rhizoids of fern gametophytes are functionally equivalent to the roots of higher plants (Kamachi *et al*., 2005). Presumably, Pb was taken up by rhizoids. Besides, in this study the black tree gametophytes were submerged in Pb solutions. Therefore, it is likely, Pb also entered the green leaf-like part of the gametophytes directly.

4.2 Three-Month-old black tree fern plants used in Pb uptake studies

In 3-month-old-black tree fern plants, treatments with increasing concentrations of $Pb(NO_3)_2$ resulted in an increase in metal accumulation in roots followed by shoots and leaves.

However, particularly at higher concentrations Pb (250 and 500 µM), the plants show significant changes in physiological and morphological characters, such as reduction in plant growth. In addition, the Pb accumulation also resulted in ultrastructural changes in leaves, shoots and roots of black tree fern plants (see 4.6).

Numerous studies have reported that heavy metal concentration in the plant body is a function of heavy metal content in the environment. When the soil is contaminated with heavy metals, the plants growing in it will take up the metal via their root system (Nwosu *et al*., 1995). In polluted air, plant leaves can play an important role in immobilising and accumulating heavy metals, resulting in a relatively high level of heavy metal concentrating in the aboveground tissues (Sawidis *et al.*, 1995; Xiong 1998). In this study, the black tree fern roots were directly exposed to Pb contamination, while the shoots and leaves were not. Thus, the Pb concentration in the roots was more closely related to that of the medium than in the shoots and leaves. The crucial factors determining Pb distribution in different plant tissues may lie in the Pb translocation process in the plant. Wheeler and Hinchey (1971) found that uranyl ions were absorbed rapidly by oat roots and bound initially in large quantities in cell walls, intercellular spaces and in root secretions. The absorption of zinc by bean roots, which is also initially rapid, is unchanged by respiratory inhibitors and considered to be primarily a non-metabolic process (Joseph *et al*., 1971). There is apparently no similar study on initial Pb uptake by plant roots.

Root surfaces, which have developed specifically to absorb elemental nutrients from soil and pore water, have extraordinarily large surface areas and high-affinity chemical receptors (Salt *et al*., 1995). In the process of absorption, root surfaces bind many elemental pollutants as well as nutrients. For example, Indian mustard *(Brassica juncia)* can rapidly concentrate Cd (II), Ni (II), Pb (II), and Sr (II) into root tissues at levels 500 times greater than those in the liquid medium in which they are growing (Salt *et al*., 1999). Sunflower roots concentrate uranium 30,000-fold from water contaminated with dilute but

highly toxic concentrations of this oxyanion (Dushenkof *et al*., 1997). The present study also indicated that a black tree fern roots might operate in a similar way as far as Pb absorption is concerned.

It was observed in the present study that black tree fern roots seem to be more sensitive to Pb than shoots. Similar results have also been observed in *Sesamum indicum* (Kumar *et al*., 1995), *Sinapis alba* (Fargasova, 1994), lettuce, and radish (Nwosu *et al.,* 1995). One of the explanations for roots to be more responsive to toxic heavy metals in environment might be that roots were the specialized absorptive organs so that they were affected earlier and subjected to accumulation of more heavy metals than any of the other organs (Xiong, 1998).

While plants are known to concentrate Pb in the roots, Pb translocation to the shoots is normally very low (Jones *et al*., 1973; Malone *et al*., 1974). Actively growing roots provide a barrier, which restricts the movement of Pb to the aboveground parts of plants (Jones *et al*., 1973). This restricted movement of Pb may explain why Pb concentrations in shoots were relatively less than in the roots. This point of view is further substantiated by a recent finding which showed that significant Pb translocation to the shoots of Indian mustard was observed only at relatively high concentrations of Pb in the hydroponics solution and after the Pb-binding capacity of roots was partially saturated (Kumar *et al*., 1995). Compared to many plants where relatively much less Pb is translocated to the shoot, black tree fern plants are unusual as evidence obtained have suggested that a large amount of Pb appeared to have translocated to the leaves, particularly in response to 250 µM Pb for 21 days.

In natural soils, these adsorption processes are orders of extent less efficient than in liquid medium (Raskin *et al.,*1997) because roots must compete for nutrients with diverse particulate soil materials (e.g. clays and humic acids). In contrast to the behavior of citrate, most organic chelators increase metal ion uptake and translocation in plants. In response to nutrient metal ion deficiencies, plants secrete phytosiderophores such as mugenic and avenic acids. These metal-chelators increase the bioavailability of metals that are otherwise tightly bound to the soil and help to carry them into plant tissues.

Interestingly, in plants there is an Al-resistance mechanism that results in the exclusion of Al from the root apex through the release of Al-binding ligands such as organic acids (Jones, 1998). When these ligands are released into the rhizosphere, they effectively chelate Al^{+3} and reduce its entry into the root. Furthermore, in several species, including snapbean, maize, wheat, and buckwheat, increased Al resistance is correlated with Al-dependent citrate, malate, or oxalate release (Pellet *et al*., 1995). It would be interesting to determine whether Pb-treated black tree ferns might have increased levels of synthesis and excretion of organic acids in roots and what effects these might have as far as Pb uptake is concerned.

Tight binding of Pb to soils and plant material at least partially explains the relatively low mobility of this metal in soils and plants. Pb binding to clay and organic matter and its inclusion in insoluble precipitates make a significant fraction of Pb unavailable for root uptake by field-grown plants.

Several mechanisms, including at the anatomical, biochemical and physiological levels (Salt *et al.*, 1995), can be concerned in heavy metal translocation in plant, thus complicating the metal accumulation and distribution in above ground tissues in the case of soil contamination. The black tree fern plants accumulated an unusually high level of Pb in their tissues during experimental period. This means that, as Salt *et al.*, (1995) pointed out, the plants can be compared to solar driven pumps, which can extract and concentrate Pb from the medium. The visual non-specific symptoms of Pb toxicity are rapid inhibition of root growth, stunted growth of the plant and chlorosis (Burton *et al*., 1984). When Pb enters the cells even in small amounts it produces a wide range of adverse effects on physiological processes. Pb phytotoxicity leads to inhibition of enzyme activities, distressed mineral nutrition, water imbalance, and change in hormonal status and alteration in membrane permeability. These disorders disturbed normal physiological activities of the plant. At high concentrations, Pb finally may lead to cell death (Ernst, 1998; Seregin and Ivanov, 2001).

To study the phytotoxic effects of Pb on the popular vegetable Chinese cabbage (*Brassica pekinensis* Rupr.) via depression of nitrogen assimilation, pot culture experiments with three concentrations Pb (0, 4, and 8 mmol/kg dry soil) were carried out (Xiong, 2006). Adverse effects of Pb on nitrogen assimilation and plant growth were found. The effect of

Pb was also shown by the progressive decline in shoot biomass with increasing Pb concentrations in plant shoots and in the soil. However, at the treatment levels used in this study, Pb did not induce visible toxic symptoms. The lowest concentration Pb treatment stimulated chlorophyll b content but did not influence chlorophyll a content. The results suggested that the toxicity of Pb to the plants occurred at least partly via depression of nitrogen assimilation (Xiong, 2006).

Stunting is a commonly observed growth response in a wide range of plants grown in metal-laden soils (Foy *et al*., 1978). The reduced shoot biomass, root biomass and decreased leaf biomass of Pb-treated plants can be due to toxicity of the metal to the plant, antagonism with other nutrients in the plant, or inhibition of root penetration in the soil. In this study, root penetration was not variable since plants were grown in relatively small volumes of containers, with $Pb(NO_3)_2$ prepared in nanopure water. It is possible that the stunting and anthocyanin pigmentation in leaves of Pb-treated black tree fern plants could be ascribed to a deficiency of nutrients like phosphorous. Pb is known to form insoluble complexes with phosphorous (Johnson *et al*., 1977; Johnson and Proctor, 1977). Similar anthocyanin pigmentation and inhibited growth have also been found in a greenhouse study involving Indian mustard treated with 500 µg/ml Pb. In this study, shoot and root biomass of Pb-treated plants were reduced 6% and 44%, respectively compared to the control plants.

In a comparative study, it was demonstrated that five species accumulated Pb, but they exhibited differential ability to take up Pb from solid media and to transport and concentrate Pb in their shoots. Although sunflower accumulated the greatest amount of Pb in its roots, it was least effective in translocation Pb to its shoots (Baker and Brooks, 1989). Three *Brassica* species growing on metalliferous soils were found to accumulate large amounts of heavy metals in their roots and shoots (Baker and Brooks, 1989). It must be noted however, that although radish showed the ability to accumulate Pb, its sensitivity to high levels (500 µg/ml) of Pb as evidenced by its reduced biomass, may limit its potential in any phytoextraction effort.

Baker and Brooks, (1989) compared the accumulation of metals by tolerant and non-tolerant clones of several plant species and concluded that there is no pattern regarding tolerance and accumulation. Both shoot and root concentrations are equally variable even

when only one particular metal is considered. However, at least in some cases, it is clear that increased tolerance is related to greater accumulation of metals.

Compartmentation in the vacuole and chelation in the cytoplasm are among the most significant mechanisms proposed to be related to metal accumulation by plants. Metal transport from the cytosol to the vacuole is studied as an important mechanism of both metal tolerance and accumulation in plants. For this reason, much work has been devoted to investigating subcellular localization of metals in hyperaccumulators (Vázquez *et al*., 1994; Küpper *et al.*, 2000; Hirschi *et al*., 2000; Krämer *et al*., 2000; Sarret *et al.*, 2002). The ultrastructural localization of Pb in black tree fern material was also investigated and will be discussed later.

4.3 Phytoremediation potential of black tree fern plants

Phytoextraction is a moderately new technology and is very dependent on plant and soil factors. In addition, it is a promising remediation technology, it is in its early stage of improvement and very little information is available related to site clean up from start to finish. Therefore, a full understanding of the physiology, biochemistry, heavy metals uptake, of metal hyperaccumulator plants is still required (Brooks, 1998).

Plants through several natural biophysical and biochemical processes can remediate heavy metal adsorption, transport and translocation; hyperaccumulation; or transformation and mineralization (Richard, 2000). For example, many elemental pollutants enter plants through nutrient transport systems. The degradation of endogenous toxic organics or their sequestration in vacuoles also protects plants from toxic xenobiotics. In many cases, the overexpression of subsisting plant genes or transgenic expression of bacterial or animal genes is required to enhance these natural properties (Richard, 2000).

Another natural mechanism that offers exciting phytoremediation possibilities is the transformation of toxic elements into relatively harmless forms. Many elements (e.g. arsenic, mercury, iron, selenium, chromium) can exist in a variety of states, including different cationic and oxyanionic species and thio- and organo-metallics. These forms vary widely in their transport and accumulation in plants and in their toxicity to humans and

other life forms. Mercury offers perhaps the best-understood example of the dangers inherent in one particular species of a heavy metal (Richard, 2000).

Hyperaccumulation is usually defined as the concentration of a metal ion to >0.1–1% of the dry weight of the plant (Baker, 1999). At these concentrations the recovery of metals from the plant tissues is potentially economical. This more detailed definition includes plants that accumulate metals in aerial tissues other than leaves, which might be useful for phytoextraction as well (Reeves & Baker, 2000).

As a plant-based technology, the success of phytoextraction is inherently dependent upon several plant characteristics. The two most important characters include the ability to accumulate large quantities of biomass rapidly and the ability to accumulate large quantities of environmentally important metals in the shoot tissue (Kumar *et al*., 1995; Cunningham and Ow, 1996; Blaylock *et al.,* 1997). The combination of high metal accumulation and high biomass production results in the most metal removal.

The success of phytoextraction depends on the selection of suitable species that produce large biomass and also tolerate and accumulate toxic. Phytexteraction of Pb polluted soils has been especially challenging since few species have been identified. Black tree fern has fast–growing with high biomass and it has high potential to accumulation Pb in its tissues.

The brake fern *(Pteris vitata)* seems to have an amazing ability to accumulate high concentrations of arsenic (Ma *et al.,* 2001). This type of fern can accumulate in its shoots up to 95% of the arsenic taken up from the soil. It is however, also well known that buckwheat is also able to accumulate a large amount of Al in the shoots (Ma & Rao, 1997). Moreover, the mechanisms have been well recognized by Shen *et al*., (1997). Tamura *et al.,* (2005) have recently discovered that common buckwheat *(Fagopyrum esculentum Moench)* can naturally accumulated up to 4,200-µg g-1 of Pb in the shoot. Common buckwheat is the first known Pb hyperaccumulator species with high biomass productivity. This relevant finding specifies this species as an excellent candidate for remediating Pb-contamination. The relationship between metal hyperaccumulation and tolerance is still question. Some authors have proposed that there is no correlation between these traits (Baker & Walker, 1990; Baker *et al*., 1994), while others suggest that hyperaccumulators possess a high tolerance to metals (Chaney *et al.,* 1997). Evidence obtained in this study

suggests that a black tree fern plant possesses some of the critical characteristics of a Pb hyperaccumulator. Further studies should be carried out to investigate and confirm this.

In general, black tree fern plants used in this study had a strong capacity to accumulate Pb in its roots, shoots and leaves. Pb and the growth of plant can be stimulated by low Pb supplies.

The black tree fern has broad ecological amplitude. The preferred growing conditions this damp, moist partially shaded places. This plant ought to be able to withstand temperatures down to about 25°F before the cold would begin to do serious damage. It is very adaptable and hardy even in less than ideal conditions. It can grow in the majority of areas on New Zealand, Fiji and Polynesia, and in other countries. Moreover, it has the characteristics of a fast growth rate, large biomass, strong roots, which make it is easy to be harvested mechanically. Overall, the potential of black tree fern as a relatively powerful species in extraction of Pb via its root system is promising and worthy of further investigation.

Therefore, there is a great potential for using black tree fern in the remediation of Pb-contaminated soils, water and it provides a new resource for exploring the biochemical mechanisms of Pb hyperaccumulation and detoxification.

4.4 Calcium and Pb toxicity

The toxicity symptoms seen in the presence of excessive amounts of heavy metals may be due to a range of interactions at the cellular/molecular level. Toxicity may result from the binding of metals to sulphydryl groups in proteins, leading to an inhibition of activity or disruption of structure, or from the displacing of an essential element resulting in deficiency effects (Van Assche and Clijsters, 1990). In addition, heavy metal excess may stimulate the formation of free radicals and reactive oxygen species, perhaps resulting in oxidative stress (Dietz *et al.*, 1999).

Calcium is an essential plant nutrient. As the bivalent cation (Ca^{2+}), it is involved in the structure of the cell wall and membranes, interacts with inorganic and organic anions in the vacuole, and as acts an intracellular messenger in the cytoplasm. Calcium enters plant cells through Ca^{2+}-permeable ion channels in the plasma membranes (White, 2000).

Calcium treatment could have an implication for Pb accumulation by plant (Broyer *et al.,* 1972).It was first found that Pb uptake over a 4 h period by excised barley roots was initially physicochemical in nature, then metabolic, but both phases were unaffected by the addition of 50 um Ca^{2+}. Simon (1978) demonstrated an increase in the tolerance indices of various plant species including *Funaria ovina* in solutions of Pb when calcium was added. Several other reports (Krzeslowska *et al*., 2004) apparently confirm the same effect for other heavy metals.

Addition of calcium to Pb solution resulted first in better growth of protonemata of moss *Funaria hygrometrica* (Basile *et al*., 1995). They were longer and contained more cells. This suggests that calcium protected cells from Pb toxicity. Protective effect of Ca^{2+} on Pb^{2+} toxicity may be the result of competition at the entry level. Ca^{2+} and Pb^{2+} are similar in ionic radius, oxidation state and electric charge. They may compete in entering the cells. It has been shown lately that non essential elements such as Pb and Cd use channels and transporters of the plasma membrane which are normally functioning in uptake of other essential for the cell ions such as Mg (for reviews see Clemens 2002). For Pb^{2+}, probably calmodulin-dependent cyclic nucleotide-gated channel is involved. Thus, presence of Ca^{2+} in the medium may limit Pb^{2+} uptake and transport and in this way may lower Pb concentration in the protoplast (Kawasaki and Morutsugu 1987). It has been found recently that Pb^{2+} uptake into *Chlorella vulgaris* cells was completely inhibited in the presence for Ca^{2+}. Ca^{2+} blocked also Pb^{2+} transport into the *Oryza sativa* roots and Pb^{2+} toxicity on root growth (Kim *et al.,* 2002).

Pb toxicity causes a decrease in mitotic activity in cells divisions (Samarakiewicz and Wozny, 2004). In meristematic cells, Pb causes modifications of the mitotic process (Wierzbicka, 1989) and inhibition of cytokinesis (Wierzbicka, 1989). Calcium ions can neutralize toxic effects of this metal on cell divisions as it was shown, for example, in pea cells. Addition of Ca with Pb in the medium caused an increase of the mitotic index, decrease of number of cells with modification, for example, chromosome fragmentation or chromatin bridges. Moreover, calcium concentrated the alterations in the structure of mitotic spindle, chromatin and chromosomes (Marme, 1985).

Preliminary evidence obtained in the present study also supports that Ca^{2+} might have a beneficial effect on Pb toxicity in black tree fern gametophytes system. This system seems an interesting material for further studies into the protective effect of Ca^{2+}.

4.5 Nitric oxide and Pb toxicity

The NO donor SNP as a signaling molecule is involved in various key roles, such as disease resistance, stomatal movement, response to abiotic stresses, as well as various developmental processes in plants (Delledonne *et al.,* 1998). However, information about whether or how NO is involved in modulating Pb toxicity response is very limited.

Pb toxicity causes excess ROS, which cause peroxidation of essential membrane lipids in the plasmalemma or intracellular organelles, resulting in damage of cellular membranes. NO plays a protective role for cell activities, membrane integrity, and chlorophyll content (Laspina *et al.,* 2005)

NO could act as a toxic compound or an important signal or bioactive molecule to scavenge ROS, depending on its location and timing of production, and concentrations in cells. A non-enzymatic reduction of nitrite to NO was found recently in the apoplast of the barley aleurone layer. In the barley aleurone layers, NO is proposed to modify the redox state in the cell, act as an antioxidant, and delay cell death (Beligni *et al*., 2002). On the other hand, NO can be removed by activating detoxification.

Cytotoxic and cytoprotective effects of NO in animals and plants have been reported (Beligni and Lamattina, 2001). NO acts as an antioxidant and can delay senescence, prevent ROS-induced cytotoxicity and slow chlorophyll breakdown.

The exposure of pea roots to Pb resulted in an increased superoxide anion production (Yamasaki *et al.,* 2001). This increase probably intensified the activity of the antioxidative system, which led to a subsequent decrease in the production of O_2 following a 48-hrs Pb^{2+} exposure. The increased generation of ROS in other plants exposed to heavy metals has been observed, e.g. in Cd-stressed potato plants (Patel *et al.,* 2000). Preliminary observation not shown here confirmed that Pb exposure also resulted in excess ROS in black tree fern gametophytes.

Preliminary results obtained in the study suggest that NO can protect black tree fern gametophytes from Pb toxicity. This gametophyte system is an attractive alternative to many other complex higher plant tissues for studies into the precise cellular and molecular mechanisms underpinning how NO can protect plant cell from Pb toxic effect. Of immediate interests for future research is to examine the relationship between NO and antioxidative defenses in Pb-treated black tree fern gametophytes.

4.6 Ultrastructural observations of Pb-treated plant cells

4.6.1 Ultrastructural changes in roots

An analysis of the use of transmission electron microscopy for the localization of Pb within plant tissues examined the possible dissolution and/or redistribution of Pb during the chemical preparation of tissues for ultrastructural observations. (Antosiewicz and Wierzbicka, 1999). It was found that Pb loss during specimen preparation in Pb-treated roots was minimal. On average, 96.2% of the Pb remained in the tissues after preparation. No difference was observed in the distribution of Pb deposits in unstained specimen and stained specimen.

The ultrastructural results from a study of the black tree ferns roots provide the evidence that at higher concentrations of Pb (250 and 500 µM Pb), the toxic symptoms of root cells mainly included disintegration of cell organelles, disruption of membranes, detachment of plasma membrane from cell walls, and formation of multivesiculate bodies in the cytoplasm.

In ultra-thin root sections of Pb-treated tree fern, (*Cyathea medullaris*) large Pb deposits were observed in remnants of the cytoplasm. Drops or clumps of strong Pb deposits were seen combined to the cell wall, scattered in the middle lamella and accumulated in the intercellular space (Plate 14a and Plate 14b).

Plants possess a range of potential cellular mechanisms that may be involved in the detoxification of heavy metals and tolerance to metal stress. However, higher Pb concentrations will result in structural changes in roots, stems and leaves and modified physiological and morphological characters.

Early studies reported that the occurrence of electron-dense deposits in vacuoles and appearance of small vesicles in cytoplasm seemed to be common features of metal-stressed plants. The presence of metal-bearing deposits in the vacuoles and vesicles is related metal detoxification and tolerance of plants treated with heavy metals (Bazzaz *et al.,* 1974).

Cellular processes leading to the sequestration of toxic ions and consequently protecting key metabolic sites (Wierzbicka, 1995) play an important role in tolerance to heavy metals, especially to Pb. Analysis of the distribution of Pb deposits in the root cells of black tree fern showed the presence of Pb in structures where it has also been found in other plants: cell walls and vacuoles (Samardakiewicz and Wozny, 2000; Glinska and Gabara, 2002). Pb is accumulated in a path that is not toxic to root metabolism in these cellular compartments. Pb is observed much less frequently in the nucleus and nucleolus. The presence of Pb deposits in the protoplast at sites other than those where Pb is detoxified causes toxic effects in the root (Wierzbicka, 1995; Glinska and Gabara, 2002). In the analyzed roots section of mature black tree fern plants, numerous Pb deposits were found in between the cytoplasm and cell wall. This is similar to that described in *Allium cepa* roots as the second stage of cell damage by Pb (Wierzbicka, 1984). One of the main sites of nontoxic heavy metal sequestration is the cell wall. This applies particularly to Pb, since it has the highest affinity for the polygalacturonic acids that are part of the cell wall (Wierzbcka, 1995; Seregin and Ivanov, 2001). Numerous authors have described large deposits of Pb in this compartment (Samardakiewicz and Wozny, 2000). The plant cell wall is the main site for detoxification of heavy metals in plants (Hayens, 1980; Allan and Jarrell, 1989). At exposure to 500 μM Pb and with the longer Pb exposure time, Pb location in the cell wall increased considerably.

There are two pathways for solubilized heavy metals in media to enter a plant. They are apoplastic (extracellular) and symplastic (intracellular). In an ultrastructural study of Pb uptake in radish, Lane and Martin (1977) reported that Pb transport in the root was possible through either the apoplast or the symplast.

The nature of this deposition suggests a combination of both apoplastic and symplastic to Pb transport. The heavy deposition of Pb around the intercellular space and within the cell walls in suggestive of apoplastic transport and Pb within structures in the cytoplasm indicates a symplastic transport.

Accumulation of Pb in cell walls may be a passive process related to apoplastic transport of it and to its retention by numerous cell wall components. Pb may also be accumulated as the result of being removed from the protoplast through plasmotubules (Wierzbicka, 1995). Transport of Pb has also been observed in the dictyosomal vesicle, together with material for the building of cell walls (Malone *et al*., 1974).

4.6.2 Ultrastructural changes in shoots

After Pb has entered the root, it is either stored in the root or translocated to the shoots. The mechanisms involved in transport of heavy metals in plants are not well understood.

Interestingly, in some ultra-thin sections of black tree fern shoots treated with Pb, some fungal hyphae were found. These hyphae appeared to have accumulated Pb. The significance of this is not known at present. However, the mycorrhizal species *Suillus bovinus* and *Thelephora terrestris* both protected *Pinus sylvestris* against Cu toxicity. The amount of Cu retained by the two fungi varied considerably (Van Tichelen *et al*., 2001).

The mechanism, by which solutes, having moved symplastically from root epidermal cells to the parenchyma cells of the vascular cylinder, enter the vessels or tracheids of the xylem, is postulated to be via some type of highly selective active-carrier transport, as opposed to facilitated diffusion (Raven *et al*., 1999).

Pb moves predominantly into the root apoplast and thereby in a radial manner across the cortex and accumulates near the endodermis. The endodermis acts as a partial barrier to the movement of Pb between the root and shoot. This may in part account for the reports of higher accumulation of Pb in roots compared to shoots (Jones *et al.*, 1973; Verma and Dubey, 2003). Future studies may determine whether transport of Pb from the root to shoot follows a similar route in a black tree fern plant.

4.6.3 Ultrastructural changes in leaves

The present results show that Pb-treated black tree sporophytes show pronounced modifications in the ultrastructure of leaf cells. The modifications predominantly concern the cytoplasm, although thickening of cell walls was also observed, as previously reported (Stefanowska *et al*., 1999).

Higher concentrations of Pb cause cell injury and disturb the barrier function of the plasmalemma as well as the selective permeability of the plasmalemma and tonoplast. Seregin *et al.,* (2004) found that a significant amount of Pb was retained at the surface of plasmalemma rather than in the cell walls. Pb enters the injured cells together with compounds such as the procion dyes, which do not enter the undamaged cells (Seregin *et al*., 2004).

TEM analysis displayed a disruption in chloroplast ultrastructure with an irregular thylakoid system. The damage in thylakoid structure suggests important disturbances in the metabolic function of the organelles affecting chlorophyll biosynthesis, photosynthesis and activities of redox enzymes probably leading to a decrease in growth (Fryer, 1992).

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APPENDIX 1

Murashige and Skoog (1962) Stock Solution

MS Major Salt Stock (10 X)	**1 Litre**	**2 Litres**
NH_4NO_3	16.5 g	33 g
KNO_3	19 g	38 g
$CaCl_2.2H_2O$	4.4 g	8.8 g
$MgSO_4.7H_2O$	3.7 g	7.4 g
KH_2PO_4	1.7 g	3.4 g

MS Minor Salts Stock (100 X)	**1 Litre**	**2 Litres**
KI	0.083 g	0.166 g
H_3BO_3	0.620 g	1.240 g
$MnSO_4.4H_2O$	2.230 g	4.460 g
$ZnSO_4.7H_2O$	0.860 g	1.720 g
$CuSO_4.5H_2O$	0.0025 g	0.005 g
$CoCl_2.6H_2O$	0.0025 g	0.005 g
$Na_2MoO_4.5H_2O$	0.025 g	0.050 g

MS Organic stock (100 X)	**500 mL**	**1 Litre**
myo-inositol	5 g	10 g
nicotinic acid	0.025 g	0.05 g
pyroxidine-HCl	0.025 g	0.05 g
thiamine- HCl	0.005 g	0.01 g
glycine	0.1 g	0.2 g

Iron stock (100 X)	**200 mL**
A: $FeEDTA.7H_2O$	1.39 g
B: Na_2 $EDTA.2H_2O$	1.865 g

Nutrient Solution (Full Strength)	**250 mL**	**500 mL**	**1 Litre**
Major Salts Stocks (10 X)	25 mL	50 mL	100 mL
Minor Salts Stocks (100 X)	2.5 mL	5 mL	10 mL
Organics (100 X)	2.5 mL	5 mL	10 mL
Iron stock (100 X)	2.5 mL	5 mL	10 mL
Sucrose (3 %)	7.5 g	15 g	30 g

Adjust pH to 5.8
Adjust to volume with dH_20 (bring up to 1 litre)

Agar	2 g	4 g	8 g
Nutrient Solution (1/10 Strength)	**250 mL**	**500 mL**	**1 Litre**
Major Salts Stocks (10 X)	2.5 mL	5 mL	10 mL
Minor Salts Stocks (100 X)	0.25 mL	0.5 mL	1 mL
Organics (100 X)	0.25 mL	0.5 mL	1 mL
Iron stock (100 X)	0.25 mL	0.5 mL	1 mL
Sucrose (3 %)	7.5 g	1.5 g	3 g
Adjust pH to 5.8			
Adjust to volume with dH_20 (bring up to 1 litre)			
Agar	2 g	4 g	8 g